STUDIES INTO THE FUNCTION OF THE MICROSOMAL GLUTATHIONE S-TRANSFERASE.

This thesis is my own composition and describes a project carried out by myself; experiments performed by other people are appropriately acknowledged.

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"I weep for you," the Walrus said:
"I deeply sympathise."
"With sobs and tears he sorted out
Those of the largest size,
Holding his pocket-hankerchief
before his streaming eyes.

Lewis Carroll (1832-1898).

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Abstract.

The glutathione S-transferase (GSTs) are a multi-gene family of proteins found in all aerobic-life forms; this suggests a fundamental role. These proteins catalyse the nucleophilic attack of the sulphur ion of the tripeptide of glutathione (γ-L-glutamyl-L-cysteinyl glycine) on a wide range of electrophilic substrates. The conjugation of glutathione to reactive compounds leads ultimately to their detoxification and hence a protective role has been proposed for these enzymes.

The majority of the work in this field has focused on the cytosolic GSTs, however a distinct membrane bound GST, the microsomal GST, also exists. Unlike the cytosolic enzymes the microsomal GST holds a latent activity, which can be rapidly increased by covalent modification of the protein. The microsomal GST is located in the hydrophobic environment of a number of membranes and it possesses a single cysteine residue which is susceptible to covalent modification by reactive metabolites and oxidised thiol compounds.

In order to investigate the role of the microsomal GST in the metabolism of a range of putative substrates an *in vivo* model was created. The microsomal GST was successfully expressed in *Saccharomyces cerevisiae* under the control of the PGK promoter. Subcellular fractionation of the yeast demonstrated the microsomal GST to be present in the membrane fraction. In addition the membrane fractions were used to demonstrate the biochemical activity of the expressed protein towards the model GST substrate; 1-chloro-2,4-dinitrobenzene.

The results from the cytotoxicity tests, conducted on the yeast model, demonstrated the involvement of the microsomal GST in the metabolism of hexachloro-1,3-butadiene and 1,2-dibromoethane. Hexachloro-1,3-butadiene is a well characterised *in vitro* substrate for this enzyme, where as 1,2-dibromoethane is not. However there is evidence to suggest that the P450 metabolite of 1,2-dibromoethane is the substrate for the microsomal GST. These results from the cytotoxicity experiments demonstrate the value of such recombinant expression systems in the study of drug metabolism.

Expression of the microsomal GST in *Escherichia coli* under the T7

RNA polymerase system allowed sufficient protein to be isolated for raising antiserum. The *E.coli* expression system potentially offered the means to produce large amounts of recombinant protein, which would allow the activation mechanism and catalytic site to be fully investigated. The protein expressed under the control of the T7 RNA polymerase was trapped, in an inactive form, in inclusion bodies. Although the microsomal GST could be recovered from inclusion bodies in a soluble form no enzyme activity could be measured. Another approach was taken to express the protein in a soluble form and thereby avoid damaging the enzyme on denaturation. This was achieved by fusing the microsomal GST to a soluble cytosolic protein, which could be be removed by a specifically engineered proteolytic site, after purification. The construction of a number of chimeric proteins sufficiently altered the properties of the microsomal GST to allow its stable expression in *E.coli.* Studies are continuing to develop a reconstituted system in which the properties of the microsomal GST could be studied further.

Finally, the hormonal regulation of the expression of the microsomal GST was studied in the rat and the mouse. Using a range of animal models, the pituitary gland was shown to regulate the expression of the mouse liver microsomal GST, but not the rat form of the enzyme. Further studies implicated growth hormone in mediating the effect of the pituitary gland in the mouse liver. In common with other drug metabolising enzymes the highest level of expression of the microsomal GST was observed in the liver. In addition the microsomal GST was observed in zone 3 of the liver, which is shared by other xenobiotic metabolising enzymes. The highest level of extrahepatic tissue expression of the microsomal GST was observed in the lung. This observation supports the hypothesis that the microsomal GST may be involved in the protection of membranes from oxidative attack.

Contents.

Abbreviations.

ACTH	adrenocorticotrophin hormone.
ADH	alcohol dehydrogenase.
ADP	adenosine diphosphate.
AFB_1	aflatoxin B_1.
AMP	adenosine monophosphate.
Amp.	ampicillin.
APAP	N-acetyl-ρ-amino phenol.
ARE	antioxidant responsive element.
3-AT	3-amino-1,2,4-triazole.
ATP	adenosine triphosphate.
BCNU	1,3-bis(2-chloroethyl)-1-nitosourea.
BHA	2(3)-*tert*-4-hydroxyanisole.
BHT	2(3)-*tert*-4-hydroxytoluene.
BPO	benzoyl hydroperoxide.
BSA	bovine serum albumen.
^0C	celcius (degree).
Ca^{2+}	calcium ion.
cal.	calorie.
cAMP	cyclic adenosine monophoshate.
CAT	chloramphenicol acetyl transferase.
CCl_4	carbon tetrachloride.
cDNA	complementary DNA.
CDNB	1,2-chloro-2,4-dinitrobenzene.
Chloro.	chloramphenicol.
CIAP	calf intestinal alkaline phosphatase.
Cl	chloride.
CM	carboxymethyl.
CTAC	cetyltrimethylammonium.
DAS	down stream activating sequences.
DBE	1,2-dibromoethane.
DCE	1,2-dichloroethane.
DCNB	1,2-dichloro-4-nitrobenzene.

DEM	diethylmaleate.
DEPC	diethylpyrocarbonate.
DNA	deoxyribonucleic acid.
DPPD	N,N'-diphenylenediamide.
DTT	dithioerthritol.
$\Delta\Psi$	membrane potential.
EDTA	ethylenediaminetetraacetic acid.
FPLC	fast pressure liquid chromatography.
GAL	galactose.
γGT	γ glutamyl transferase.
GH	growth hormone.
GHRH	growth hormone releasing hormone.
Glr	glutaryl.
Gn-HCl	guanidine hydrochloride.
GS^-	thiolate ion.
GSH	glutathione (γ-L-glutamyl-L-cysteine glycine).
GSSG	oxidised glutathione.
GST	glutathione S-transferase.
H_20_2	hydrogen peroxide.
HBcAg	hepatitis B core antigen.
HCBD	hexachloro-1,3-butadiene.
4HNE	4 hydroxyl-2,3-trans nonenal.
HPLC	high presure liquid chromatography.
HRP	horse raddish peroxidase.
Hsp 70	heat shock protein 70.
IgG	immunoglobulin G.
IPTG.	isopropyl-β-D-thiogalactopyranoside.
j	joules.
K	potassium.
k_2	bimolecular rate constant.
Kano.	kanomycin.
k_{cat}	first order rate constant.
KDa	kilodalton.

K_m	Michaelis constant.
La^{3+}	lathanum ion.
LB	L-broth.
lit.	little (mice).
Log.	logarithmic.
M	molar.
3,5-Me_2NAPQI	3,5-dimethyl-N-acetyl-ρ-benzoquinone imine.
Mg^{2+}	magnesium ion.
min.	minute(s).
MMLV	Moloney murine leukemia virus.
mRNA	messenger RNA.
Na	sodium.
NAD(H)	nicotinamide adenine dinucleotide (reduced form).
NADP(H)	nicotinamide adenine dinucleotide phoshate (reduced form).
NAPQI	N-acetyl-ρ-benzoquinone imine.
NEM	N-ethylmaleimide.
NMR	nuclear magnetic resonance.
$O_2^{\cdot -}$	superoxide radical.
$OH^{\cdot}$	hydroxyl radical.
14KDa OMM	14KDa outer mitochondrial membrane protein.
ORF	open reading frame.
P	pellet (derived by centrifugation).
P450	cytochrome P450.
P450 reductase	NADPH-dependent cytochrome P450 oxido-reductase.
PAGE	poly acrylamide gel electrophoresis.
PAH	polycyclic aromatic hydrocarbon.
PBS	phosphate buffered saline.
PCR	polymerase chain reaction.
PDGF	platelet derived growth factor.
PFK	phosphofructokinase.
PGK	phosphoglycerate kinase.
PGM	phosphoglycerate mutase.

RBS	ribosomal binding site.
RNA	ribonucleic acid.
rRNA	ribosomal RNA.
S9	superntant derived from centrifugation of cell lysate (liver) at 9 000g.
SDM	site directed mutagenesis.
SDS	sodium dodecyl sulphate.
SMC/IGF1	somatomedin C/ insulin like growth factor.
SN	supernatant (derived by centrifugation).
SOD	superoxide dismutase.
T3	triiodothyronine.
T4	thyroxine.
TBST	Tris buffered saline tween.
TCDD	2,3,7,8-terachloro-benzo-ρ-dioxin.
TNB	2,4,6-trinitrobenzene.
TNM	tetranitobenzene.
TPA	12-O-tetradecanoyl phorbol 13-acetate.
TRE	TPA responsive element.
TRH	thyroxine releasing hormone.
TRIS	tris(hydroxymethyl)aminoethane.
tRNA	transfer RNA.
UV	ultraviolet.
XGal.	5-bromo-4-chloro-3-indocyl-β-D-galactosidase.
XRE	xenobiotic responsive element.

One and three letter abbreviations for the amino acids.

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Abbreviations for the nucleic acids.

Adenine	A
Cytosine	C
Guanine	G
Thymine	T
Uracil	U

Chapter 1. Introduction.

1.1 General Overview.

The glutathione S-transferases (GSTs) are a multi-gene family of proteins found in all aerobic-life forms; this suggests a fundamental role. These proteins catalyse the nucleophilic attack of the sulphur ion of the tripeptide glutathione [γ-L-glutamyl-L-cysteinyl glycine (GSH)], on a wide range of electrophilic substrates. In evolutionary terms glutathione may have became abundant as a biomolecule when oxygen became abundant (Mannervik 1986). Reactive oxygen species created during respiration can attack the carbon double bonds of polyunsaturated fatty acids in the cell. This can initiate a chain reaction known as lipid peroxidation, which is detrimental to cells. The GSTs have been shown to metabolise products and intermediates of this process, and hence a protective role has been proposed (Ketterer and Meyer 1989; Ketterer *et al* 1990). The concept of GSTs having a protective role within the cell has been extended to their role in the detoxification of xenobiotics (reviewed by Hayes and Wolf 1988). Many products from the chemical and pharmaceutical industry have been found to be substrates for these enzymes, as have naturally occurring compounds such as aflatoxin B_1.

The GSTs are subject to tissue-specific and sex-specific expression (Hatayama *et al* 1986; Igarashi *et al* 1983,1985). Also regulation of levels of expression of these proteins can be altered by xenobiotics or hormonal control. In recent years much attention has focused on chemotherapeutic treatment of tumours, it has shown that acquired resistance to these agents is associated with an increase in GST expression. Investigation into the regulatory mechanisms has been pursued in order to gain insight into the function of these proteins and their putative protective role in the cell.

One of the most striking features of these enzymes is a very broad substrate range. The fact that GSTs comprise a multi-gene family partly explains this phenomenon, but also individual enzymes can often catalyse a large number of reactions.

Using the model substrate for GST, 1,2-dichloro-4-nitrobenzene (DCNB), subcellular fractionation of rat liver lead to the observation that 82%

of GST activity was recovered in cytosol, 4% in the microsomes, 5% in the mitochondria and 5% in the nuclei (Booth *et al* 1961). At this time DCNB was the model substrate for measuring GST activity, however this has now been replaced by 1-chloro-2,4-dinitrobenzene (CDNB) (Habig *et al* 1974). Friedberg *et al* (1979) showed that the activity in the microsomes was not due to cytosolic contamination or the translation of cytosolic GST on ribosomes. The purification (Morgenstern *et al* 1982) amino acid sequencing (Morgenstern *et al* 1985) and finally cloning of the cDNA (DeJong *et al* 1988) have elucidated the existence of a membrane form of glutathione S-transferase, named the microsomal GST. Unlike the cytosolic GST there appears to be only one enzyme encoded on a single gene (DeJong *et al* 1988).

The sequence data reveals that there is no significant homology between microsomal GST and the cytosolic GSTs. The best reported alignment of the microsomal GST with any of the cytosolic forms was with the maize GST (Mannervik and Danielson 1988). However the homology was still low and the authors appeared not to allow for the membrane spanning domain of the microsomal GST. In fact, in terms of homology the cytosolic GSTs show more homology with glutaredoxin than the microsomal GST (Jornvall and Persson 1986). Based on similarities in amino acid sequence cytosolic GST have allowed them to be divided into four classes, alpha, mu, pi and theta. The sequence homology within a class is greater than 60%, also a high degree of homology is observed between classes across species. The classes of cytosolic GSTs and the appropriate nomenclature is shown in Table 1.1.

However, even before the sequences were published there were obvious differences to separate microsomal GST and cytosolic GST. At the level of quaternary structure level, the cytosolic enzymes exist as homo or heterodimers, each subunit binding one molecule of glutathione and the kinetics being a mixture of the two (Mannervik and Danielson 1988). The microsomal GST exists as a trimer (Morgenstern *et al* 1982 and Boyer *et al* 1986); the monomer has a molecular weight of 17kDa, whereas the cytosolic is in the range 25-29kDa.

Most of the work into the catalytic mechanism has been performed on the cytosolic GSTs. The catalytic centre of the GST consists of a two binding

Table 1.1 Cytosolic glutathione S-transferase nomenclature.

Table 1.1a Cytosolic glutathione S-transferase nomenclature in the rat.

"Y" designation.	Numerical designation.	Apparent MWT. on SDS-PAGE.	Family.
Ya	1	25 500	alpha
Yb_1	3	26 300	mu
Yb_2	4	26 300	mu
Yc	2	27 500	alpha
Yf or Yp	7	24 800	pi
Yk	8	25 000	alpha
Yl	10	25 700	alpha
Yn_1 or Yb_3	6	26 000	mu
Yn_2	9	26 000	mu
Yo	11	26 500	mu
-	5	~26 300	theta
-	12	~ 26 300	theta

Table 1.1b Cytosolic glutathione S-transferase nomenclature in the human.

GST isoenzyme.		Family	Subunit MWT on SDS-PAGE.
B_1B_1	ε	alpha	25 900
B_1B_2	δ	alpha	25 900
B_2B_2	γ	alpha	25 900
	μ	mu	26 700
	ψ	mu	26 600
	ϕ	mu	26 700
	π	pi	24 800
	θ	theta	24 800-25 900

Taken from: Glutathione S-transferases and drug resistance (Eds. Hayes JD. Pickett CB. and Mantle TJ.),1990, Taylor and Francis, New York and London. Theta class isoenzymes taken from: Meyer *et al* (1991) Theta, a new class of glutathione S-transferase purified from rat and man. Biochem. J. **274** 409-414.

sites: a G site where glutathione binds and a hydrophobic site (H site) were acceptor substrates can be accommodated (Mulder *et al* 1990). The H site has a very low specificity which explains the diversity of GST substrates (Armstrong *et al* 1990). The catalytic mechanism of the microsomal GST have not been so extensive, however the same broad catalytic mechanism has been proposed for this enzyme as the cytosolic forms (Andersson and Morgenstern 1992). In some respects this is surprising as so little sequence homology is observed between the two different enzymes forms.

Both enzymes utilise binding energy to deprotinate the sulphur of glutathione, which results in the thiolate ion (GS^-) and this can be viewed spectroscopically in both enzymes as a Messeinhmer complex (Graminski *et al* 1989b; Andersson and Morgenstern 1992). Based on chemical modification studies, histidine was suggested as the amino acid in the active site (G site) responsible for the formation of this species in both enzymes (Aswathi *et al* 1987; Andersson and Morgenstern 1992). However, the crystal structure of the π GST (Reinemer *et al* 1991) has revealed tyrosine 7 to interact with the GS^- in the case of the cytosolic enzymes. It is thus possible that the microsomal and cytosolic GSTs have different active sites.

However, it is clear both enzymes share the ability to conjugate glutathione to electrophilic substrates, although, the kinetic parameters reveal anomalies between the two. The Km for glutathione is an order of magnitude higher for the microsomal GST (2mM), than the cytosolic GSTs (0.1-0.2 mM). Studying specific activity of the model substrate, CDNB, gives a value in the range of 33μM/min/mg for the cytosolic GST and 2μM/min/mg for the microsomal GST (Morgenstern and DePierre 1985). However some cytosolic GSTs, such as the theta class have no activity for the model substrate. In general appears to be the case that the microsomal enzyme has lower specific activities than the cytosolic enzymes.

In evolutionary terms the lack of homology and differences in protein properties make it questionable that the microsomal GST has a common ancestor with the cytosolic forms, although it is possible the microsomal GST could have arisen by a convergent pathway. The fact that the microsomal GST appears to be unrelated to the cytosolic GSTs in both physical and evolutionary terms makes it possible that the microsomal GST may also

have a distinct function. Conversely the microsomal GST shares common substrates with the cytosolic GST, including peroxidation products (Ketterer *et al* 1990: Mosialou and Morgenstern 1989) and similar inhibitors, such as steroid metabolites (Tahir *et al* 1985; Mosialou and Morgenstern 1990) makes it tempting to suggest a common role.

The factors regulating an enzyme can be a pointer as to their function within the cell. For example induction of GST isoenzymes by xenobiotic substrates could provide protection for the cell by enhancing the cells capacity for detoxification (reviewed by Hayes and Wolf 1988). Unlike the cytosolic GSTs, the expression of the microsomal GST cannot be raised transcriptionally by inducers of drug metabolising enzymes such as phenobarbital, 3-methylcholanthrene and *trans*-stilbene oxide (Morgenstern *et al* 1980; DeJong *et al* 1988). The unique feature of this protein is a latent activity which can be released on treatment with sulphydryl reagents such as N-ethylmaleimade (NEM) (Morgenstern *et al* 1983a). A single cysteine at position 49 is the site of covalent modification (Morgenstern *et al* 1985). Secondly similar activation can be observed by tryptic cleavage - a lysine 41 (Morgenstern *et al* 1989). It is important to note that these are chemical modifications produced *in vitro*, and it is unclear how these findings relate to the function of the microsomal GST *in vivo*.

Naturally occurring reducing agents such as oxidised glutathione (GSSG) can cause an increase in the activity of microsomal GST (Anyia and Anders 1989a; Nisho and Ito 1989), but again these observations have been made *in vitro*. Agents that disrupt thiol status and bring about an increase in GSSG have been shown to bring about the increase in microsomal GST activity, following treatment of whole animals or cultures cells (Botti *et al* 1982; Masukawa and Iwata 1986; Wies *et al* 1992).

Lipid peroxidation is an example of a form of toxic insult which results in the disruption of thiol status bringing about an increase in glutathione disulfide (Reed, 1990). The elevation of cytosolic Ca^{2+} seems to be the ultimate step in this form of toxicity (Orrenius 1985) and both calcium and thiol homeostasis perturbances are inter-related (Reed 1990). The products of lipid peroxidation have been shown to be substrates for both the cytosolic and microsomal GST (Ketterer *et al* 1990; Mosialou and Morgenstern 1990), the location of the microsomal GST would favour its role as a protective

mechanism against membrane attack. However, it is also worth considering the plethora of diffuse mechanisms the cell has produced to afford protection against this major form of insult.

The microsomal membrane is also the site of a number of drug metabolising enzymes notably the cytochrome P450 (P450) system. Recent work has demonstrated that many of the reactive metabolites produced by this system can not only be metabolised by the microsomal GST, but can in fact activate this enzyme by covalent modification of the sulphydryl group (Haenen *et al* 1990; Wallin *et al* 1991; Lundqvist *et al* 1992a and b). It is noteworthy that UDP glucuronysyl- and sulpho-transferases have also been shown to be activated by sulphydryl reagents, under certain conditions, *in vitro* (Morgenstern and DePierre 1985). It has been argued that "Glutathione S-transferase(s) localised in the organelle can truly be said to be in the mainstream of drug metabolism" (Morgenstern *et al* 1980).

The unique activation mechanism of this enzyme may hold the answers to the function of this enzyme and merits some careful consideration.

1.2 Activation and Regulation of the microsomal Glutathione S-transferase.

Indication of the cytosolic GST involvement in drug metabolism can be seen from large induction of these enzymes by xenobiotics such as phenobarbital, 3-methylcholanthrene and *trans*-stilbene oxide. The induction is thought to occur at the transcriptional level causing increase synthesis of the mRNA, leading to increased expression of the protein. These substances are not inducers of the microsomal GST (Morgenstern *et al* 1980), although some slight induction (44%) has been with 2(3)-*tert*-butyl-4-hydroxyanisole, as compared with a 14-fold induction to cytosolic GSTs (Benson *et al* 1979 and 1978) and a 50% increase with the peroxisome proliferator acetylsalicylic acid (Morgenstern *et al* 1990). The latter may not be surprising as the microsomal GST is located in the peroxisomes. Unlike enzymes which are activated by transcriptional mechanisms the microsomal GST seems to hold a latent activity which can be rapidly increased by covalent modification. Examples of factors that mediate the activation of the

Table 1.2 Agents capable of activating the microsomal glutathione S-transferase.

Author.	Experimental conditions.	Compound (Conc).	Location of the microsomal GST. and increase in activity (fold).			Reactive species.	Proposed activation mechanism.
Thiol modifying compounds.							
Morgenstern	*in vitro*			microsomes			
et al (1979)		NEM (1mM)		7.77		NEM	Thiol-disulphide exchange
		IAA (10mM)		3.96		IAA	Thiol-disulphide exchange
Morgenstern	*in vitro*	DTNB (1mM)		4.50		DTNB	Thiol-disulphide exchange
et al (1980)		DTP (1mM)		3.9		DTP	Thiol-disulphide exchange
Aniya *et al*	*in vitro*		Enzyme - GSH	Enzyme +GSH	microsomes		
(1989a)		Cystamine (5mM)	3.29	3.24	5.36	Cystamine	Thiol-disulphide exchange
		Diamide	2.79 (4mM)	9.61(0.5mM)	5.37(4mM+GSH 1mM)	GSH	Thiol-disulphide exchange
		GSSG (10mM)	0.72	-	3.20	GSSG	Thiol-disulphide exchange
		NEM (1mM)	10.42	13.95	5.66	NEM	Thiol-disulphide exchange
Nishino	*in vitro*			microsomes.	GSSG/GSH ratio		
et al (1989a)		GSSG (0.1mM)		2.20	-	GSSG	Thiol-disulphide exchange
		GSSG (0.1mM)/GSH(1mM)		0.76	0.1	GSSG	Thiol-disulphide exchange
		GSSG (1mM)		6.9	-	GSSG	Thiol-disulphide exchange
		GSSG (1mM)/GSH (1mM)		1.1	1	GSSG	Thiol-disulphide exchange
		GSSG (10mM)/GSH (1mM)		2.6	10	GSSG	Thiol-disulphide exchange
		GSSG (10mM)		6.9	-	GSSG	Thiol-disulphide exchange
Masukawa	*in vivo*			microsomes.			
et al (1986).	rats	phorone (100mg/kg)		2.45		GSSG	Thiol-disulphide exchange
		DEM (500mg/kg)		1.77		GSSG	Thiol-disulphide exchange
	in vitro	cystine (1mM)		2.58		Cystine	Thiol-disulphide exchange
		homocystine (1mM)		2.53		Homocystine	Thiol-disulphide exchange
		GSSG (1mM)		2.03		GSSG	Thiol-disulphide exchange
		cysteine (1mM)		1.02			
		homocysteine (1mM)		0.98			
		GSH (1mM)		0.77			
Lundqvist	*in vivo*			microsomes			
et al (1992a)	isolated	phorone (1mM)		2.62		GSSG	Thiol-disulphide exchange
	hepatocytes	DEM (2mM)		1.00		GSSG	Thiol-disulphide exchange

Author.	Experimental conditions.	Compound (Conc).	Location of the microsomal GST. and increase in activity (fold).		Reactive species.	Proposed activation mechanism.
Reactive metabolites.						
a). Direct action of the compound.						
Botti *et al*	*in vivo*		microsomes.			
(1982)	rats	DBE (0.4mmol/kg)	1.28		DBEor metab(GST/P450 med.)/GSSG?	cov. mod?
		CCl_4(4mmol/kg)	1.59		CCl_4 or metab(P450 med.)?	cov. mod?
Lundqvist	*in vivo*	DBE (1mM)	1.40		DBEor metab(GST/P450 med.)/GSSG?	cov. mod?
et al (1992b)	isolated	CCl_4(1mM)	0.87			
	hepatocytes	CDNB (0.3mM)	1.61		CDNB or metab?	cov. mod?
		tBuOOH (0.5mM)	1.12		tBuOOH metab/GSSG	cov. mod?
Wies *et al*	*in vivo*		microsomes			
(1992)	isolated	NAPQI (160μM)	3.39		NAQPI/GSSG?	cov. mod. via -SH
	hepatocytes	3,5Me_2NAPQI (210μM)	1.75		GSSG?	cov. mod. via -SH
		mBBr (4mM)	5.00		mBBr/GSSG	cov. mod. via -SH
	in vitro		microsomes			
		NAPQI (250μM)	3.34		NAQPI/GSSG?	cov. mod. via -SH
		3,5Me_2NAPQI (250μM)	4.05		GSSG?	cov. mod. via -SH
b). P450/P450 reductase dependent generated metabolites.						
Aniya *et al*	*in vitro*		microsomes			
(1989b)		NADPH (regen.)	1.80		H_2O_2, $O^{\cdot -}_2$	cov. mod. via -SH
		NADPH +NA (0.1mM)	4.00		H_2O_2, $O^{\cdot -}_2$	cov. mod. via -SH
Lundqvist	*in vitro*	NADPH +NA (1mM)	1.60		quinone form	cov. mod.
et al (1992a)						
Haenen *et al*	*in vitro*	α-methyl dopa (1mM)	microsomes			
(1988)		+NADPH (2mM)	2.84		quinone form	cov.mod.
		+tyrosinase (5U/ml)	1.78		quinone form	cov.mod.
		methyl ortho benzoquinone (1mM)	2.87		quinone form	cov.mod.
Wallin *et al*	*in vitro*		microsomes	enzyme		
(1991)		phenol + NADPH (5mM)	1.7	1.00	quinone form	cov.mod.
		benzoquinone (5mM)	2.11	8.00	quinone form	cov.mod.
		1,2,4 benzoteriol (5mM)	2.18	6.00	quinone form	cov.mod.

Author.	Experimental conditions.	Compound (Conc).	Location of the microsomal GST. and increase in activity (fold).		Reactive species.	Proposed activation mechanism.
b). ADH dependent metabolism.						
Haenen *et al*	*in vitro*		microsomes	enzyme		
(1988b).		Allyl alcohol (5mM)	1.00	-	acrolein	cov. mod. via -SH
		acrolein (5mM)	2.20	5.00	acrolein	cov. mod. via -SH
	in vivo rats.	Allyl alcohol (1.1mol/kg)	1.9		acrolein	cov. mod. via -SH
c). Reactive oxygen species.						
Aniya *et al*	*in vitro*		microsomes	enzyme		
(1992)		H_2O_2	1.72 (0.05mM)	15 (60mM)	H_2O_2, $O^{\cdot-}_2$	dimerization
Lundqvist *et al* (1992a)		H_2O_2 (10mM)	0.97			
Aniya *et al*	*in vitro*			microsomes		
(1993).	Whole liver.	Ischem/reperf. (90min/30min)		1.2	H_2O_2, $O^{\cdot-}_2$	dimerization
		Ischem/reperf. (90min/60min)		1.3	H_2O_2, $O^{\cdot-}_2$	dimerization
		3-AT (0.2%w/v)+Ischem/reperf. (90min/60min)		1.76	H_2O_2, $O^{\cdot-}_2$	dimerization
		3-AT (0.2%w/v)		1.32	H_2O_2, $O^{\cdot-}_2$	dimerization
		Perfusion with H_2O_2 (1mM).		1.25	H_2O_2, $O^{\cdot-}_2$	dimerization
Proteolysis.						
Morgenstern	*in vitro*		microsomes	enzymes		
et al (1989)		trypsin (1mg/ml)	5.0	10.0	protease activity	cleavage at lys41
Lundqvist *et al* (1992a)	*in vitro*	xanathine oxidase (60mU/ml)		4.45	protease activity	cleavage
Removal of endogenous inhibitors						
Boyer *et al*	*in vitro*		microsomes			
(1982)		phosphatidyl-choline vesicles (11mg/ml)	3.93		Lipid	absorb inhib. poss. LP products.
Nishino *et al* (1989)	*in vitro*	bovine sera Factor V (5μM)	2.00		protein	absorb inhib. poss. LP products.

Table 1.2 continued.

Author.	Experimental conditions.	Compound (Conc).	Location of the microsomal GST. and increase in activity (fold).	Reactive species.	Proposed activation mechanism.
Radiation					
Boyer *et al* (1986)	*in vitro*	(3-6 mega rads)	microsomes 1.5-2.0	Radiation?	Alter oligometric structure.
Heat.					
Anyia *et al* (1989c).	*in vitro*	(50°C)	microsomes 2.6	Unknown	Unknown, does not Cystine 49 (-SH).

Abbreviations.
NEM: N-ethylmaleimide; IAA: iodoacetamide; DTNB: 5,5'-Dithiobis(2-nitrobenzoic acid); DTP: 2,2'-dipyridyl disulfide; GSH: glutathione; GSSG: glutathione disulphide; DEM: diethylmaleate; DBE: 1,2-dibromoethane; CCl_4: carbon tetrachloride; CDNB: 1-chloro-2,4-dinitrobenzene; tBuOOH: t-butylhydroperoxide; NAPQI: *N*-acetyl-ρ-bezoquinone imine; 3,5Me_2NAPQI: 3,5-dimethyl-*N*-acetyl-ρ-bezoquinone imine; mBBr: monobromobimane; NADPH: reduced nicotinamide adenine dinucleotide phosphate; NA: noradrenaline; 3-AT: 3-amino-1,2,4-triazole (catalase inhibitor).
metab.: metabolism; med.: mediated; cov.mod.: covalent modification; Ischem/reperf.: ischemia/reperfusion; absorb inhib. poss. LP products: adsorbtion of inhibitors, which are possibly lipid peroxidation products.

microsomal GST are illustrated in Table 1.2 and summarised in Figure 1.1.

The idea that proteins can be regulated by reversible thiol-disulfide exchange is not limited to the microsomal GST, other examples have been cited (Ziegler 1985). Cystamine and diamide both activate the purified enzyme and this is reversed by dithiothreitol (DTT) (Anyia and Anders 1989a). The activation with diamide is potentiated by GSH, this is thought to be due to the fact that diamide catalyses the formation of protein-SSG in microsomal GST. Cystamine, however, reacts directly with the enzyme. Glutathione disulfide will not activate the purified enzyme, but requires the enzyme to be in the membrane. In addition, the increase in activity is enhanced at 37^{o}C, which indicates a prerequisite for endogenous factors to be present. Two such mechanisms of thiol modification can be proposed from these results, one of direct covalent modification and a second of enzymatic protein mixed disulfide formation.

Animal experimentation provides evidence that such mechanisms can be operative *in vivo*. It has been demonstrated that injection of rats with the glutathione depletor phorone (diisopropylidene acetone) leads to a two-fold increase in microsomal GST activity which is maximal after 24 hrs (Masukawa and Iwata 1986). GSH was found to be depleted in two hours, which lead to the stimulation of glutathione synthesis. After 24 hrs both GSH and GSSG concentrations were doubled, but the final ratio remained unchanged from the initial values. In the intervening period thiol status was perturbed and protein mixed thiol levels increased; this is concomitant with increased microsomal GST activity. Again the disulfide compounds increased the activity which was reversible by DTT, but the reduced counterparts had no effect.

Nishino and Ito (1989) showed that the activation of the microsomal GST, in rat liver microsomes, by GSSG was affected by the concentration of GSH present, and that the GSSG/GSH ratio was important. A maximal activation of 6.9 fold could be achieved in the presence of 10mM GSSG, however was reduced in the presence of 1mM GSH and 10mM GSSG to 2.6 fold when the GSSG:GSH ratio was 10. If the ratio fell below 1 then no stimulation or a decrease in activity was observed. The decrease in activity at high GSH concentrations might suggest that a small portion of the enzyme is already activated in untreated microsomes. Under physiological

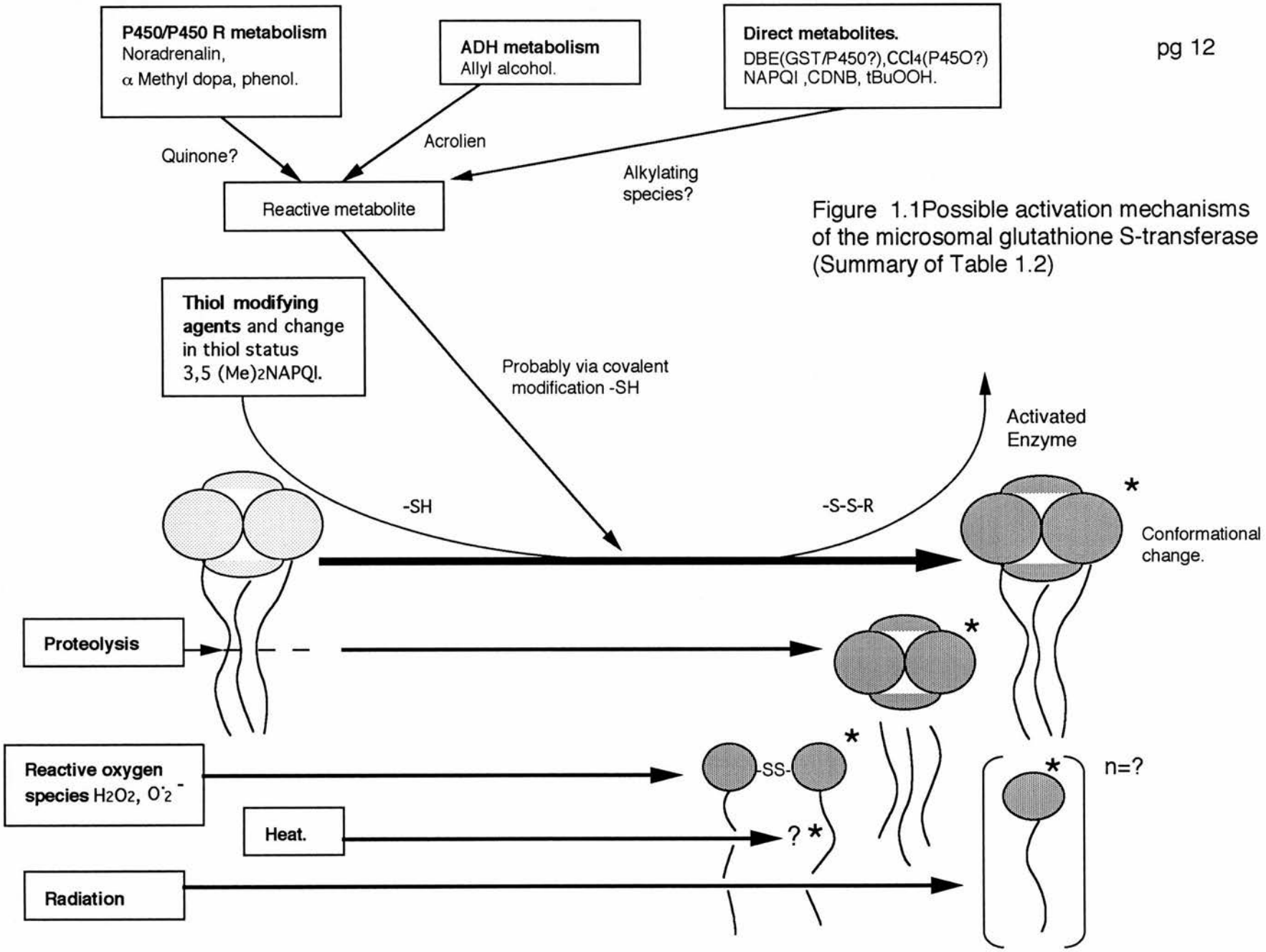


Figure 1.1Possible activation mechanisms of the microsomal glutathione S-transferase (Summary of Table 1.2)

conditions the ratio between the oxidised and reduced forms of glutathione is a consideration when assessing *in vivo* regulation of the enzyme.

A recent study showed that the activity of the microsomal GST was increased in isolated hepatocytes treated with phorone, but the activity in the isolated microsomes or the purified enzyme was not increased by such a treatment (Lundqvist and Morgenstern 1992b) To investigate the possibility that thiol-disulfide interchange had taken place, pretreatment of the hepatocytes with a thiol depletor diethylmaleate (DEM) was undertaken. However the increase in activity brought about by the addition of phronone was not inhibited DEM, nor did DEM result in activation of the microsomal GST on its own. The increase in activity of the phorone-treated microsomes was only slightly decreased by DTT treatment. The authors could offer no explanation for the observations with phorone and the results contradict the earlier studies (Anyia and Anders1989a; Masukawa and Iwata 1986). This certainly appears to be a controversial area other evidence exists to suggest thiol-disulfide exchange is a mechanism of activation and is discussed later.(Section 1.4)

The non sulphydryl compounds, 1,2-dibromoethane (DBE) and carbon tetrachloride (CCl_4), cause an increase in microsomal GST activity two hours after injection into rats (Botti *et al* 1982). This is accompanied by loss of GSH and a non-competitive inhibition of cytosolic GSTs. Conjugation of DBE results in the formation of the highly reactive episulphonium ion, which binds to DNA (Van Bladderen *et al* 1980). The majority of DBE is metabolised by the P450 system (Van Bladderen *et al* 1981) the products of which bind to proteins (Guengrich *et al* 1980). This may explain the loss of cytosolic GST activity and concomitant increase in microsomal activity within the same time period. In addition, disruption to thiol status could result in the formation of mixed protein thiols in both cases. Interestingly, CCl_4 causes loss of P450 activity, suggesting P450 metabolism of this compound to produce a reactive product towards proteins. In a more recent study the activity of the microsomal GST in isolated hepatocytes was raised on DBE treatment, but not by CCl_4 (Lundqvist and Morgenstern 1992 b)

Not only does the microsomal GST share the same location as the phase I monooxygenase system, but a large number of xenobiotics are lipophilic in nature and may preferentially concentrate in membranes within

the cell. Recently there have been a number of examples of NADPH-dependent activation of the microsomal GST, however, there is some conflict in the literature as to the causative agent. Noradrenalin plus a NADPH regenerating system was shown to activate the microsomal GST, via the same mechanism as NEM (Anyia and Anders 1989b). Superoxide dismutase (SOD) and catalase partially reduced the activation implicating oxygen species, such as hydrogen peroxide or the superoxide anion, causing the activation. These species would have arisen from the P450 metabolism of noradrenalin or the P450 reductase regeneration system, which has the ability to produce reactive oxygen species by redox cycling. Further experiments have demonstrated that hydrogen peroxide can directly activate the microsomal GST (Anyia and Anders 1992). These findings are contradictory as other studies have disputed the direct involvement of the superoxide anion and hydrogen peroxide. The direct application of hydrogen peroxide or a H_2O_2 regenerating system failed to activate the microsomal GST, nor did superoxide anion radical generation (Lundqvist and Morgenstern 1992a). However, incubation of microsomes with [^{3}H] labelled noradrenalin, caused 20% of the label to be associated with the microsomal GST, following analysis on SDS-PAGE. As the microsomal GST only accounts for 3% of the total microsomal protein, the noradrenalin metabolites preferentially attack the microsomal GST (Morgenstern *et al* 1983b).

In a similar study α-methyldopa activated the microsomal GST in the presence of NADPH (Haenen *et al* 1991). The activation was inhibited by GSH, implicating the role of cysteine 49. However, horseradish peroxidase-hydrogen peroxidase-catalysed oxidation of α-methyldopa produced (−)-isoproterenol and 4-methylcatechol, which could also induce activation. This form of oxidation produces the semi-orthoquinone and the orthoquinone of α−methyldopa. Furthermore, the stable chemically synthesised 4-methyl orthobenzoquinone, alone activated the enzyme, in a manner which could not be enhanced by NEM. The 4-methyl orthobenzoquinone is unable to decompose to the semi-orthoquinone form. Earlier work had demonstrated that the P450 metabolism of α-methyldopa produced products that could covalent binding to proteins. This effect could be blocked by SOD. (Dybing

et al 1976).

Further evidence of P450-derived metabolites activating microsomal GST, comes from the metabolism of phenol (Wallin *et al* 1991). Phenol catechol and hydroquinone activate the membrane bound form of this enzyme in the presence of NADPH. The phenol metabolites benzoquinone and 124 benzentriol can both activate the purified enzyme, whereas their parent compounds cannot. The ^{14}C labelled parent compounds again preferentially label the microsomal GST when the proteins were resolved on SDS-PAGE, although the majority of the label comigrates with the P450 band.

Covalent modification of the sulphydryl group in the enzyme may explain some earlier work. Mitozantrone, an anticancer drug and a quinone, undergoes microsomal glutathione conjugation for which P450 metabolism is a prerequisite (Wolf *et al* 1986). Interestingly no activation of GSH conjugation could be observed by the addition of NEM. It may be possible that the quinone produced by the P450 had already activated the GST. Alternatively, this substrate may not be a sufficiently reactive to allow activation by a sulphydryl agent to be observed.

Xenobiotic metabolism in general produces reactive compounds, lending many opportunities for the microsomal GST to be activated. Allyl alcohol is metabolised by cytosolic alcohol dehydrogenase (ADH) to acrolein, which is generally believed responsible for the toxic effects (Haenen *et al* 1988). *In vitro* it is acrolein which will activate the microsomal GST and not the parent compound, allyl alcohol. This increase in activation of the microsomal GST can be blocked by GSH and it is not additive with respect to NEM. Allyl alcohol administered to rats activates the transferase, however the pretreatment of the animals with the cytosolic ADH inhibitor pyrazole blocks the activation *in vivo*. These two sets of experiments demonstrate that it is the toxic metabolite of acrolein which is responsible for increasing the microsomal GST activity. Acrolein and NEM both inhibit the vitamin E free radical reductase *in vitro*, another sulphydryl sensitive protein. The activity of this enzyme *in vivo* was unaffected by treatment. It is possible the sulphydryl group of the microsomal enzyme is more accessible to such thiol modifying agents.

Cysteine 49 is located C-terminally from the putative membrane

spanning domain residues 11-35 (Morgenstern *et al* 1985). This region of the microsomal GST is hydrophilic in nature and seems to be sensitive to modification. The enzyme can be cleaved at position lysine 41 by trypsin, leading to enzyme activation with similar changes in the kinetic parameters as with NEM (Morgenstern *et al* 1989). The addition of trypsin to the already NEM-activated enzyme does not activate, but renders the enzyme more susceptible to proteolytic cleavage suggesting some form of confirmational change has occurred. Treatment with other proteases brings about this effect (Morgenstern *et al* 1989, Lundqvist and Morgenstern 1992). This mechanism of protein activation is by no means novel, the most quoted being activation of zymogens by proteolytic cleavage (Blow *et al* 1971).

There are a few examples where the microsomal GST is activated by mechanisms that do not involve a covalent modification of the protein. Increased activity has been observed by heating, with a maximal value being achieved after 30 minutes at 50^{o}C (Anyia 1989). DTT did not reverse the effect, hence sulphydryl reagent or activated species are not involved, but NEM activation of the preheated enzyme was markedly less than of the native enzyme. This may suggest some confirmational change had already taken place. Radiation inactivation-treatment of proteins is used to determine the molecular size of oliogomeric proteins. When applied to the microsomal GST, surprisingly the enzyme was activated (Boyer *et al* 1986). These results suggest that a change in oligometric make up of the protein as bringing about activation.

Recent work has suggested that the microsomal GST can be activated by 1.5 fold by incubation with 0.75μM hydrogen peroxide at 37^{o}C for 10 min (Anyia and Anders 1992). This is in contradiction of the study earlier in the year by Lundqvist and Morgenstern (1992) in which neither hydrogen peroxide generating system or direct application could produce activation. However, here the authors present evidence to suggest that dimerisation takes place as a consequence of peroxide treatment and is concomitant with increased activity. Hydrogen peroxide has been known to cause S-thiolation and fragmentation in proteins. Also oxidation of proteins causes fragmentation due to confirmational changes in the proteins (Davis 1987; Dean 1987; Park and Thomas 1988; Abe and Sasaki 1989; Salo *et al* 1990)

The studies into the activation of the microsomal GST by oxidative stress have been extended to examining the changes in enzyme activity in perfused rat liver (Anyia and Naito 1993). Reperfusion followed by ischemia is well known to produce oxygen radicals (Okuda *et al* 1991; Jaesche and Mitchell 1989). When isolated liver was reperfused for 30 minutes and 60 minutes, following 90 minutes ischemia, the microsomal GST activity was increased 1.2 and 1.3 fold, respectively (Anyia and Naito 1993). The addition of DTT to microsomes, prepared from livers treated in this manner, reversed the increase in activity. Also the NEM activation of the enzyme in microsomes was lower from liver that had undergone ischemia/reperfusion, than control liver. Both these results indicate that the activation occurs via covalent modification of the -SH group in the enzyme. Immunoblotting revealed, as in previous studies, a small amount of dimerisation of the microsomal GST protein had taken place.

The authors presented two lines of evidence for oxygen species being responsible for the covalent modification of the enzyme. Firstly, perfusion of the liver with 1mM hydrogen peroxide resulted in a 1.25 fold increase in microsomal GST activity. Concomitant with this increase in activity was the loss of GSH, protein and non-protein thiols, however no increase in GSSG could be detected. Secondly, the *in vivo* administration of the catalyse inhibitor, 3-amino-1,2,4,-triazole, caused a 1.76 fold increase in microsomal GST activity, following ischemia/reperfusion, while the catalyse activity was reduced by a half.

A pattern emerges of an enzyme with a latent activity that can be rapidly utilised by covalent modification. The means of covalent modification are wide and varied and largely nonenzymatic. Clearly products of xenobiotic metabolism are instructive in such changes and thus the microsomal GST is implicated in having a function in this process Other mechanisms are indirect and result from homeostatic alterations within the cell, as mentioned briefly, lipid peroxidation could cause such changes.

1.3 The catalytic mechanism of the microsomal glutathione S-transferase, in particular reference to the activation process.

The mechanism of activation of the microsomal GST, at the protein

level, has attracted considerable interest. Unlike the cytosolic GSTs no crystal structure exists to explain the intricacies of the catalytic mechanism. However, a number of indirect techniques have been employed to elucidate to this process. In order to understand any aspect of enzymatic function, studies of kinetic parameters can give an insight into underlying mechanisms.

Not all substrates for the microsomal GST show increased activity when treated with sulphydryl reagents. Those substrates for which the microsomal GST is activatable such as CDNB, cumene hydroperoxide and p-nitrophenylacetate bear little structural resemblance, but they do have high non-enzymatic rate (Morgenstern *et al* 1988). Studies of kinetic parameters of a number of 4,chloro-3-nitrobenzene derivatives, showed that both non-enzymic bimolecular rate constants (k_2) and enzymic rate constant (k_{cat}) were linearly related to hammett resonance values of electrophilicity (σ). This is to be expected for aromatic nucleophilic substitution. Only the more reactive substrates of the group are activatable. The k_{cat} values appears to be increased on activation whereas the k_{cat}/K_m values were largely unaffected. By plotting the log k_{cat} against hammett substitution constant (σ) a straight line is formed, as expected for nucleophilic reactions the rate of the reaction is dependent on on the electron withdrawing power of the substituent ie. the σ value (Fresht 1985). The slope of the line is the Hammett ρ value, the reaction constant, upon activation this value is increased for the more reactive substrates, but a break point is observed in the series where the analogues becomes too unreactive. The ρ values for the more reactive substrates approach similar values to those found for cytosolic GSTs for this particular substrate analogue series, which suggests a change in mechanism to one that is closer to the cytosolic enzymes. The plots of the log of k_{cat}/K_m against the σ value are also linear, but the plots for activated and unactivated enzyme fall on virtually the same line. The k_{cat}/K_m values set a lower limit for the rate of association of enzyme and substrate, a mechanism of activation that affects the transition state can be ruled out, because if activation caused an increase in the rate of thiolate ion,GS^-,-formation, then all substrates would be affected. K_{cat} refers to the combined properties of all enzyme complexes and it is this parameter is increased

upon activation. Hence changes before or after the transition state must be responsible for the increase in k_{cat}. In this series of substrate analogues the steps preceding the transition state do not vary, therefore the rate at which the products are released is increased and this only affects the more reactive substrates. The authors suggested that the low Hammett ρ value of the log k_{cat} further supported the the idea that the increase in k_{cat} was brought about by an increase in product release. It would appear that activation of the microsomal GST drives the kinetic parameters near to those of the cytosolic enzymes, which is characterised by higher catalytic efficiency.

By considering all the kinetic parameters it can be seen that the changes by activation have a greater affect on the addition substrate GSH especially k_{cat}/K_m. Already studies with glutathione analogues on the cytosolic GST have allowed the G site on the enzyme to be investigated (Adang *et al* 1988b, 1989, 1990). Many of the findings and resulting models were to be confirmed by the crystal structure (Reinemier *et al* 1991).

Although GSH is the common substrate, these analogue studies highlighted a number of differences in G sites between the alpha, mu and pi class GST studied. This may partly explain different substrate activities and stereoselectivities. All the GST showed an absolute requirement for the carboxyl group of the glutamyl residues, as the 4-amino butyric-LCys-Gly were inactive. The crystal structure of π GST showed that this residue forming a salt bridge with arginine 13 in the enzyme active site, which confirmed chemical modification studies and experiments (Schasteen *et al* 1983; Sternberg *et al* 1991). Treatment of the microsomal GST with the arganine specific chemical modifying agents, phenylglyoxal (PGO) and 2,3-butanedione, caused inhibition of activity. The inhibition could be prevented by pretreatment with S-hexyl glutathione and these results implicate an arginine residue is also important in the microsomal GST active site (Anderson and Morgenstern 1992).

The amino group of the glutamyl residue may influence thiolate formation (Adang *et al* 1998a). The orientation and position on the carbonyl backbone is tolerated to a different extent by individual isoenzymes as is the overall length of the carbonyl backbone. As a generalisation alpha class isoenzymes have the most stringent requirements followed by the pi then the

mu class (Adang *et al* 1988b). The cysteinyl moiety is a requirement and orientation is of central importance to achieve competent catalysis. However the carboxyl moiety of the glycine does not seem to be critical, as glutathione esters of decarboxy analogues have high activity (Adang *et al* 1990). However, the higher k_m values observed (an order of magnitude) with such analogues suggests a role in substrate binding.

Four analogues of GSH were shown to have activity with the microsomal GST:- glutaryl-L-Cys-Gly (Glr-L-Cys-Gly), γL Glu-L-Cys-β-Ala, α L-Glu-L-Cys- Gly and α-D-Glu-L-Cys-Gly, but the latter analogue had extremely low activity (Andersson *et al* 1991). Based on these findings the G site of the microsomal is most similar to the rat alpha class enzymes 1-1 and 2-2.

More importantly the analogues were further used to probe the changes that occur in the active site upon activation. An increase in stringency and again a shift to a more "cytosolic form" was observed, as a result of activation.

Both GSH and α–L-Glu-L-Cys-Gly show an increase in k_{cat} following activation and k_{cat}/K_m values indicating that this process focuses more on GSH than the acceptor substrates. The other two analogues show a decrease or no change in k_{cat} on activation.

To examine the local changes the that G site undergoes when activated, the loss in binding energy was calculated when glutathione was replaced by an analogue (shown in Figure 1.2a). The Glr-L-Cys-Gly analogue caused a significantly larger unfavourable binding energy on activation, 9.7 kJ/mol compared with 3.2 kJ/mol in the unactivated state,when replacing GSH. This analogue is devoid of the α amino group and this deletion would appear to affect the activated state more than the unactivated form. Interestingly, the activated value is close to the unfavourable binding energy obtained with such an analogue in the cytosolic G site, 6.9-10.7 kJ/mol. The incorrectly positioned alpha amino group in the α–L-Glu-L-Cys-Gly seems to affect the unactivated and activated enzyme equally with loss in binding energy of 7.3 and 8.7 kJ/mol respectively. However, it can be seen if α–L-Glu-L-Cys-Gly is replaced by Glr-L-Cys-Gly that favourable binding energy is gained, - 4.1 kJ/mol in the

Figure 1.2a The structural changes made in GSH and the resulting changes in binding energy for the unactivated and activated enzyme. -a positive value depicts the loss of binding energy, analogue replacing GSH.

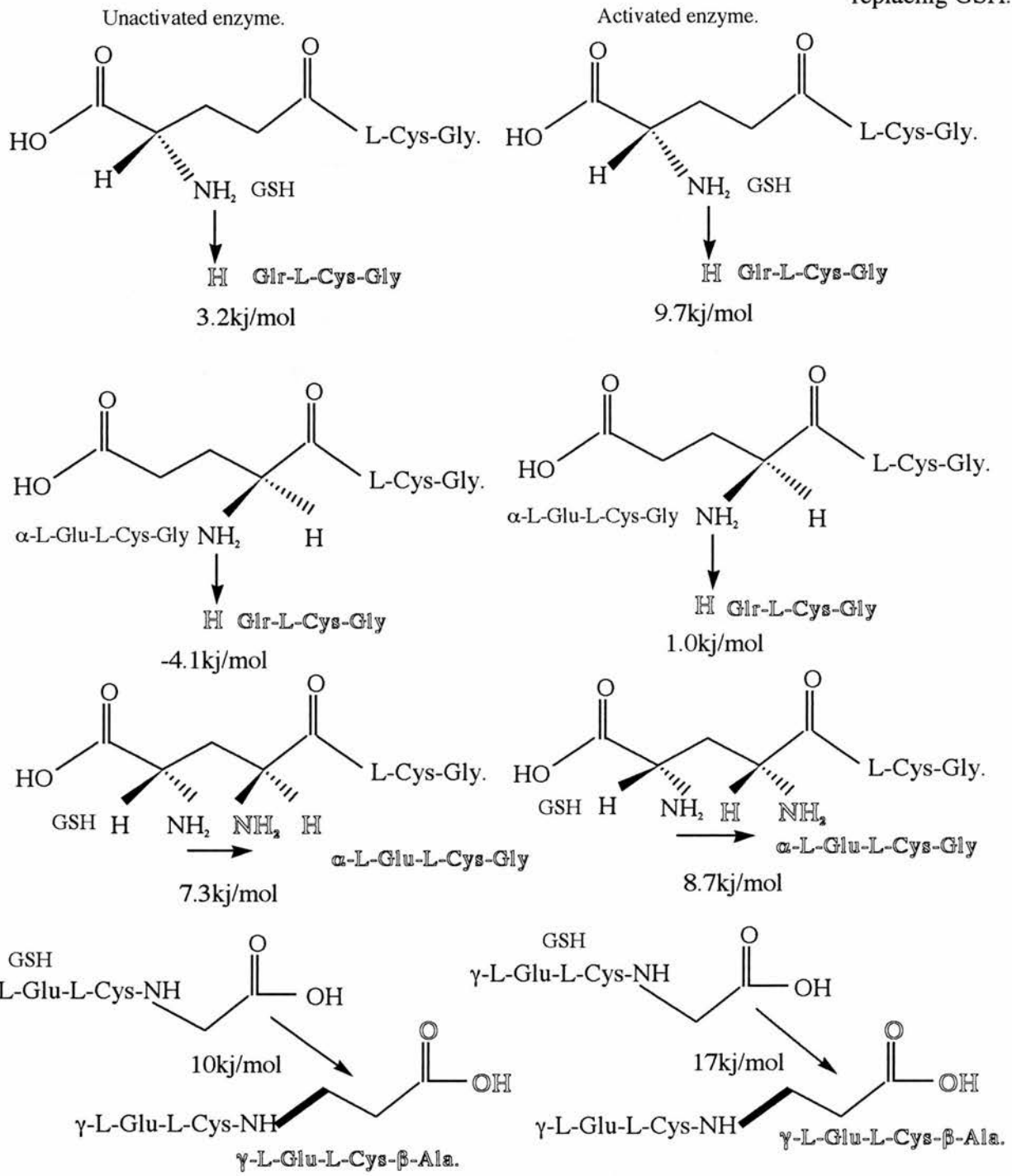


Figure 1.2b The apparent change in binding energy for glutathione and the analogues apon activation of the enzyme - a negative value depicts a gain in binding energy apon activation.

Unactivated enzyme	Activated enzyme.
Glr-L-Cys-Gly	2.3+/- 1.6 kj/mol.
α-L-Glu-L-Cys-Gly	-2.8 +/- 1.7 kj/mol.
γ-L-Glu-L-Cys-β-Ala.	2.8 +/- 0.8 kj/mol.
GSH	-4.2 +/- 1.7 kj/mol.

Taken from Andersson *et al* (1991) J.Biol.Chem.**266** 2076-2079.

unactivated state, but lost in the activated form, 10.kJ/mol, again indicating importance of the α amino residue in the activated form. The activated form of the enzyme shows a stricter requirement for the glycine carboxylate group, as replacement of GSH with γ-L-Glu-L-Cys-βAla lead to unfavourable binding of 17kJ/mol and 10kJ/mol in the activated and unactivated states, respectively. All of these changes in binding energy values again suggest a confirmational change during activation.

Activation increases the k_{cat}/K_m values for GSH; and this parameter was used to calculate the change in binding energy apon activation (shown in Figure 1.2b). A favourable increment in binding energy of -4.1kJ/mol is observed for glutathione, but for Glr-L-Cys-Cly this is only - 2.8kJ/mol upon activation, probably due to the absence of the glutamyl α group. The other two analogues show unfavourable binding energies on activation demonstrating the increased stringency of the G site with respect to those altered moieties in these analogues. It has been postulated that the unactivated enzyme does not fully utilise the binding energy from glutamyl α amino and glycine carboxyl, and activation allows the realisation of these binding energies which can then be utilised in the activated catalysis (Andersson *et al* 1991).

The fact that the k_{cat}/K_m for GSH is increased may be significant in allowing the enzyme to be operative at lower GSH concentrations than in the latent form. Such conditions occur, as illustrated in other sections (1.4 and 1.5) under toxic insult or oxidative stress. Glutathione depletion is often accompanied by increased glutathione disulfide concentration which may be instrumental in the activation of the microsomal GST.

External factors cause a confirmational change in the microsomal GST, but it should be noted that the binding of GSH to the cytosolic enzymes also has been shown to cause a confirmational change, (Prinipato *et al* 1988; Graminiski *et al* 1989b). For the cytosolic enzymes this was confirmed by the crystal structure which shows Gln 62 residue in an unfavourable position with respect to the glutamyl carboxyl residue of GSH.

The G site is thought to facilitate the formation of the active species in catalysis, which is the thiolate ion in the case of the cytosolic enzymes (Graminiski *et al* 1988). Evidence of the existence of this species comes

from comes from UV absorption studies which give a characteristic band at 239nm. Titration of this band shows the thiolate anion has a pKa 6.6, while the thiolate ion has a pKa of 8.9 in solution, the shift in pKa of 2.4 units can be translated into a binding energy of 3.3 kcal/mol which the enzyme has to achieve to bring about catalysis (Graminski *et al* 1989a). Glutamyl (D,L amino malonyl) glycine in which the -SH group is replaced with the negative COO^- group, which binds 30-90 times more tightly to the enzyme than GSH. Therefore binding energy calculations using this analogue show that 2.0-2.6 kcal/mol of the 3.3 kcal/mol must be used to stabilise the thiolate ion.

The formation of the thiolate anion is of critical importance in understanding the catalytic mechanism of the GSTs. Initially, a general base catalysed mechanism was proposed involving a basic amino acid such as arginine, lysine or histidine as seen with serine proteases and ribonuclease A (Fresht 1985). Histidine was implicated by chemical modification studies using diethylpyrocarbonate (DEPC) in the Ψ GST, human μ class (Aswathi *et al* 1987). DEPC inhibited the formation of the Meissenhimer complex, a transition state inhibitory complex, formed between GSH and 2,4,6-trinitrobenzene (TNB) (which can be observed spectrophotometrically at 450nm) in the microsomal GST (Andersson and Morgenstern 1992). Further evidence to support the idea that this residue was important in catalysis in the microsomal GST came from the fact that S-hexyl glutathione protects against DEPC inhibition. All three histidines were modified at different rates, but the loss of 90% of enzyme activity was achieved within the time period required for modification of the most reactive histidine, indicating the importance of this residue in catalysis (Andersson and Morgenstern 1992). More recent SDM experiments with the cytosolic enzymes have shown that a histidine was not critical for catalysis, although mutation of histidine 159 caused a confirmational change affecting activity, which may explain the chemical modification studies (Wang *et al* 1991; Zhang *et al* 1991).

The crystal structure of the pi class GST has shown that tyrosine 7 interacts with the thiol moiety (Reinemer *et al* 1991) This residue is conserved in all mammalian cytosolic GST (α μ and π), *Schitsosoma japonicum* and maize glutathione S-transferase (Mannervik and Danielson 1988). Tyrosine 7 is located at a site equivalent to the active site disulphides of the thioredoxins and seleno-cysteine of glutathione peroxidases

(Reinemer *et al* 1991). It is not understood how tyrosine can lower the pKa of the thiolate ion. The pKa of the tyrosine may be sufficiently decreased, to act as a general base, by the protein environment. Alternatively, it could be the enzyme active site that stabilises the thiolate anion by lowering of its pKa. An electrostatic potential generated at the N-terminal of an α helix may be capable of lowering the pKa of the tyrosine and thiol in the active site. The crystal structure reveals the N-termini of two parallel helices located near the G site. Use of such a mechanism makes evolutionary sense as a base, such as histidine, would be particularly susceptible to alkylation by electrophilic species, which are substrates for the cytosolic GSTs. As the microsomal GST is activated by alkylating agents, the involvement of a histidine in catalysis would seem rather unlikely. The use of the tyrosine modifying reagent, tetranitromethane (TNM), caused a 50% inhibition of the microsomal GST activity, which was partially blocked by S-hexyl glutathione (Andersson and Morgenstern 1992). Amino acid analysis of the TMN-treated enzyme revealed 0.5-1.5 of the 7 tyrosines residues were modified, but as this reagent also reacts with histidines the involvement of a tyrosine in the active site can not be definitely determined.

There is evidence that both cytosolic and membrane GSTs have similar catalytic mechanisms. It appears that activation drives the microsomal GST towards a "cytosolic like state" by studying kinetic parameters. However, if the mechanisms were identical a greater sequence homology might be expected, with conservation of the contact residues within the G site. However the crystal structure of the microsomal GST has not been determined and no equivalent data for the microsomal form is available. The most highly conserved region between the microsomal GST and any cytosolic enzyme is in the hydrophilic region, which would be suitable for an active site. These are residues 57-63 (NAKKFLR) that which matches residues 193-199 in rat Ya (NVKKFLQ) (DeJong *et al* 1988). However, the region in the rat Ya does not contain any G site residues and is some distance from residues involved in acceptor substrate binding (H site) residues 212-218 (Hoesch and Boyer 1989; Board and Mannervik 1991).

Based on the lack amino acid sequence homology between the microsomal and cytosolic GSTs, it is likely that the microsomal GST has evolved by a convergent pathway to perform the same catalytic process as

the cytosolic enzymes. Perhaps, given the microsomal enzymes different logistics and striking activation process its function may be subtly different to that of the cytosolic enzymes. To throw some light onto this role, changes within the cell that can bring about activation of the enzyme need to be reviewed.

1.4 Causes and effects of changes in thiol status within the cell

Thiol/disulphide exchange has been proposed as a possible *in vivo* regulatory mechanism for the microsomal GST (Anyia and Anders 1989a, Nishino and Ito 1989). Maintaining the redox status of the cell is of central importance in overall homeostatsis (Kosower and Kosower 1978). To understand the role of the microsomal GST, it is important to investigate factors which can influence, or factors which are influenced by, the redox potential of the cell.

Most of the cellular non protein thiols exists as glutathione, the majority being in the reduced form (in the mM range) and a minority being in GSSG, mixed disulphides of GSH and other cellular-thiols and minor amounts of thioethers (Kosower and Kosower 1978). GSH serves to provide a protective mechanism against chemical insult and loss of 20-30% of total GSH can impair the cell's defence against such insult. Glutathione when depleted, by either conjugative reactions or peroxidase reactions, is replenished by *denovo* synthesis or regeneration from GSSG by glutathione reductase, a cytosolic NADPH-dependant enzyme (Meister and Andersson 1983). Glutathione disulphide is also exported from the cell in order to maintain the *status quo* (Elow *et al* 1984).

In the case of oxidative insult, the cell has developed a number of defence mechanisms; the range of which may merit the severity of the problem. Within the cytosol examples of enzymes that can counteract this threat are catalyse, SOD and glutathione peroxidase (GPx).

The mitochondria account for the major source of endogenous oxidative stress, and in certain respiration states 10% of total mitochondrial GSH is turned over per minute (Sies and Moss 1978). The role of protection in this respect falls entirely to GSH, and the mitochondria contain a complete

glutathione redox system (Meredith and Reed 1982). The mitochondria are unable to export GSSG and its build up is deleterious, hence loss of mitochondrial GSH is more critical than cytosolic loss.

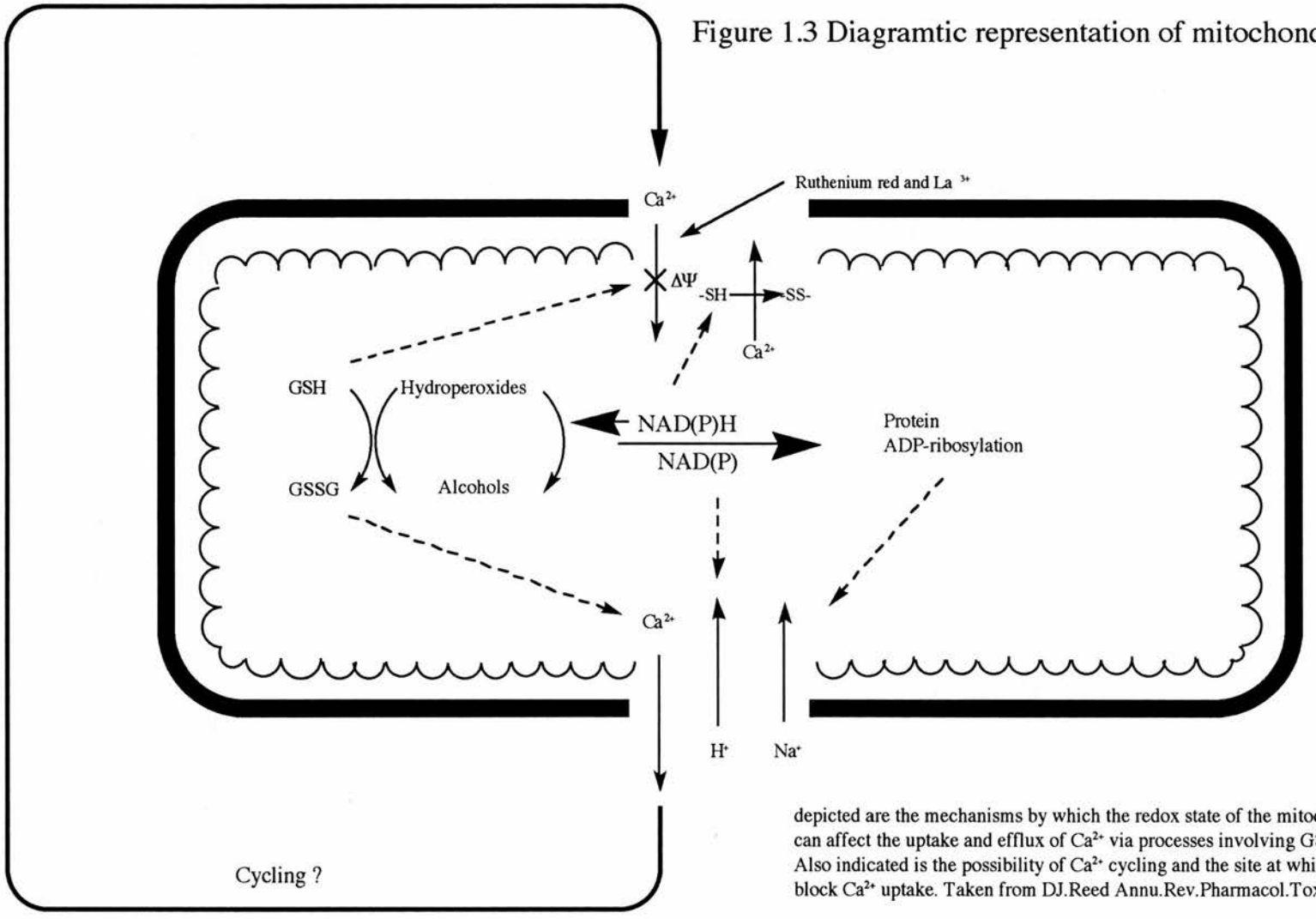
A number of chemical agents such as diamide have been used to demonstrate the effect of oxidation glutathione on mitochondria. As expected, the depletion of GSH results in the loss of protein thiols. A number of proteins are highly sensitive to such changes including Mg^{2+} and Ca^{2+}-dependent ATPases, because they require free sulphydryl groups for their activity (Bellomo *et al* 1983). The link between loss of enzyme activity and GSH depletion may be direct or due to the imbalance of pyridine nucleotides (Lehninger *et al* 1978). Oxidised intramitochondrial pyridine nucleotides, generated by GSSG reduction for example, are hydrolysed and the ADP ribose moiety can be attached to proteins. Calcium release appears to be modulated by such modifications (Vercesi *et al* 1988).

The common denominator in both these redox changes is the release of calcium form the mitochondria, this is believed to be an early step in cell injury. The mitochondria can take up calcium again via the antiport and this causes calcium cycling, a deleterious energy consuming process leading to the collapse of the membrane potential $\Delta\Psi$. Inhibitors of the antiport - ruthenium red, La^{3+}, and calcium chelators have been shown to protect against the toxic effects of oxidative stress by preventing calcium cycling (see Figure 1.3).

Artificial disruption to calcium homeostasis within the cell can be caused by the ionophore A28187, and has been used to study the mechanism of calcium toxicity. The influx into the cytosol can be created from the external medium, or from internal stores such as mitochondria and the endoplasmic reticulum when in a calcium depleted medium. The toxicity correlates well with loss of cytosolic and then mitochondrial GSH (Olafsdotti *et al* 1988). The maintenance of protein thiols by antioxidants such as N,N'-diphenylenediamine (DPPD) can increase cell viability (Pascoe *et al* 1989). However, when calcium is present in the surrounding medium, antioxidant protection is not complete, suggesting alternative mechanisms of toxicity also exist.

Similar experimental conditions can be set up to deplete cells of glutathione and investigate the effects of

Figure 1.3 Diagramtic representation of mitochondrial calcium cycling.



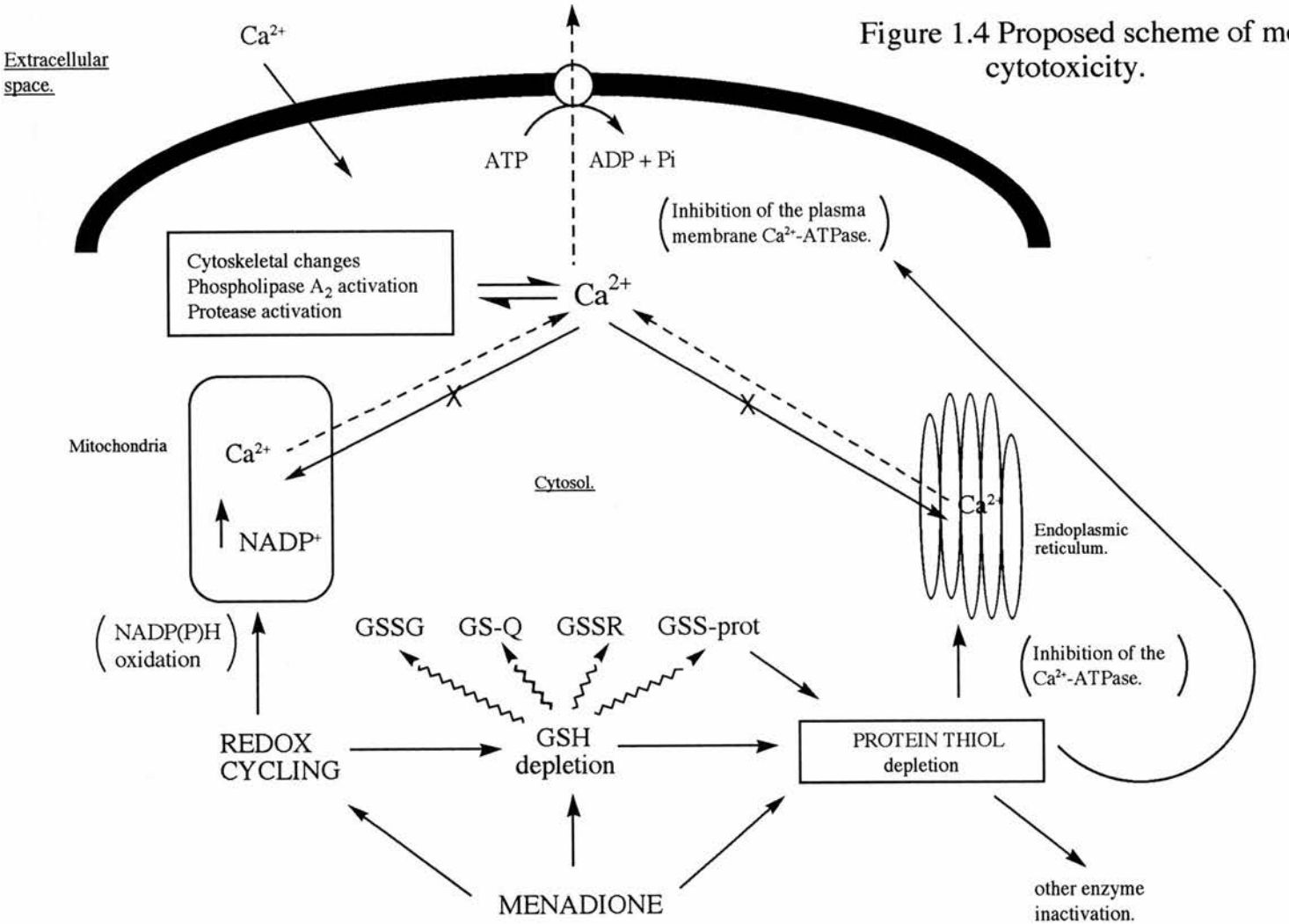
depicted are the mechanisms by which the redox state of the mitochondria [NAD(P)H/NAD(P)] can affect the uptake and efflux of Ca^{2+} via processes involving GSH and ADP-ribosylation. Also indicated is the possibility of Ca^{2+} cycling and the site at which ruthenium red and La^{3+} block Ca^{2+} uptake. Taken from DJ.Reed Annu.Rev.Pharmacol.Toxicol. **30** 603-631 (1990).

α tocopherol. In both calcium depleted and adequate cells, protein thiol content and cell viability were maintained by vitamin E irrespective of calcium status or diminished GSH levels (Pascoe *et al* 1989). What is not clear in α tocopherol-mediated protection against oxidative stress is whether calcium release is prevented by vitamin E or whether maintenance of protein thiols is enough to sustain viability despite elevated cytosolic calcium levels.

Mitochondria hold enough Ca^{2+} to raise the cytosolic levels by 5-10 fold. Calcium cycling is only one toxic consequence of thiol depletion and relates specifically to mitochondria. Various models have allowed the elucidation of the mechanism of toxicity. Menadione induced H_2O_2 production in isolated hepatocytes results in a rise in cytosolic GSSG and effects key thiols in the plasma membrane, mitochondria and endoplasmic reticulum (Orrenius 1985). The release of Ca^{2+} into the cytosol is thought to trigger calcium-dependent membrane phospholipase as illustrated in Figure 1.4 (Orrenius 1985). This may be particularly significant in the role of certain glutathione transferases and glutathione peroxidase to detoxify lipid hydroperoxides form as a result of free radical attack on membrane (Tan *et al* 1984). Also certain calcium-dependent non lysosomal proteases are activated (Nicotera *et al* 1986). This observation may be relevant to the microsomal GSTs, as proteases can activate the enzyme (Morgenstern *et al* 1989). All these changes will lead to alterations in membranes and cytoskeletal structure. For example human platelets treated with menadione undergo cytoskeletal alterations, that are concomitant with loss of thiols and ATP and increase in cytosolic Ca^{2+} concentration. However, these changes can be prevented by high levels of ATP (Mirabelli *et al* 1989). Treatment of cytoskeletal fraction isolated from menadione treated cells, with dithiothreitol (DTT), shows considerable amounts of polypeptide become associated with this structure following exposure to the quinone (Thor *et al* 1988).

Xenobiotics that directly raise GSSG, such as diamide and benzoyl hydroperoxide (BPO); or deplete GSH by conjugation; or induce oxidative stress, such as menadione or t-butyl hydroperoxide by raising GSSG; have all been shown to bring about these changes (Reed 1990). The key to the toxicity appears to be the oxidation of thiols predominantly in the mitochondria. It is interesting to note that mitochondria energised by substrate oxidation, when H_2O_2 production is maximal, show enhanced

Figure 1.4 Proposed scheme of menadione cytotoxicity.



GS-Q, menadione conjugate with glutathione; GSSR, soluble mixed disulfides containing glutathione; GSS-prot, glutathione-protein mixed disulphides.
Taken from Orrenius TIPS **FEST** S15-S20 (1985)

reaction of protein thiols with NEM. Alkylation of these thiols leads to loss of membrane integrity and selectivity for transport (le Quoc and le Quoc 1985). The thiol group in the microsomal GST appears to be preferentially alkylated (Haenen *et al* 1991 Wallin *et al* 1991 and Wies *et al* 1992), and indeed, many chemically different species preferentially attack this enzyme.

Acetaminophen (*N*-acetyl-ρ-aminophenol, paracetamol, APAP) is bioactivated by a P450 mediated oxidation resulting in the formation of *N*-acetyl-ρ-benzoquinone imine (NAQI) (Dahlin *et al* 1984). Detoxification of NAPQI occurs through the conjugation of GSH; but GSH can also react reductively with NAPQI regenerating the parent compound, APAP, with the concomitant formation of GSSG (Rosen *et al* 1984). Excessive production of NAPQI will deplete cellular GSH content and allow NAPQI to bind to cellular macromolecules, also oxidative changes in proteins and depletion of cellular protein thiol groups has been observed in APAP or NAPQI treated hepatocytes (Moore *et al* 1985)

NAPQI and its oxidising analogue 3,5-dimethyl-*N*-acetyl-ρ-benzoquinone (3,5-Me_2NAPQI), which does not bind to proteins as the reactive 3 and 5 positions have been blocked by methyl groups, were used to treat isolated hepatocytes (Wies *et al* 1992). Both compounds caused a rapid loss of protein thiols in all subcellular fractions, which correlated well with loss of cell viability. A recent technique which involves pretreatment of proteins samples with the UV fluorescent thiol reactive agent, monobromaine, allowed the thiol content of individual proteins to de viewed on SDS PAGE (Cotagreave *et al* 1988). From this study some proteins, including a 17 KDa polypeptide, appeared to be more susceptible to thiol loss than others. Amino acid determination of the 17KDa species revealed it to be the microsomal GST, and further more both NAPQI and 3,5-Me_2NAPQI activated the enzyme *in vitro*. This provides direct evidence that the microsomal GST belongs to a set of proteins that contains a thiol moiety that susceptible to modification during disruption of thiol status. Unlike many other proteins this action brings about increase activity of the enzyme, the significance of which is not fully understood, but it has been postulated that it is part of a protective mechanism against such toxic insult.

Alkyl and alkenyl halides are compounds whose toxicity is initiated by glutathione conjugation (Anders *et al* 1988). These conjugates and reactive

metabolites can covalently modify the mitochondria membrane. Oxidative species and their reduction are not a feature of this toxicity. However the covalent modification of the membrane leads to Ca^{2+} release and loss of $\Delta\Psi$ similar to that previously described. These compounds have been shown to activate the microsomal GST (Botti *et al* 1982 and Lundqvist and Morgenstern 1992b).

Disruptions to thiol status trigger a wide range of processes within the cell. Such changes lead to the covalent modification of a number of proteins (and other structures); the modifications appear to be non-enzymic in nature. The main cause of changes in thiol status appears to be oxidative stress. As the microsomal GST is one of the proteins that could be modified by the process involved, this GST may have a protective role against oxidative stress.

1.5 The involvement of the microsomal glutathione S-transferase in lipid peroxidation.

It is possible that the glutathione S-transferases have evolved with an increase in aerobic metabolism (Mannervik 1986). In addition the importance of thiol status with respect to cell viability when under oxidative stress has been noted (Reed 1990). These observations, amongst others, have implicated a role for the glutathione metabolising enzymes in protection against oxidative insult.

The specific generation of oxidative stress by polymorphonuclear leukocytes and eosinophils is part of the immune response to bacterial invasion, viral infection and tumour cell antigens. This so called "oxygen burst" comprises of the production various reduced forms of oxygen ($O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$ and hypohalous acids), which may contribute to the cell killing ability of these cells (Curnutte and Babior 1987). However cellular protective mechanisms, such as the GSTs, may well protect the surrounding tissue from damage. The oxygen burst is also thought to stimulate the production of eicosanoids, which are cell mediators associated with the inflammatory response (Lands and Pendleton 1988). The biosynthesis of the eicosanoids involves the conversion of aracidonate to leukotriene A_4, via

a number of intermediates. The conversion of leukotriene A_4 to leukotriene C_4 is a glutathione conjugation step and can be catalysed by GSTs *in vitro*. Similarly, the conversion of a arachidonate to various prostaglandin derivatives involves a number of glutathione conjugation reactions. The actual contribution of the GSTs to these reactions is at present unknown, but they may have an endogenous role in these processes (Ketterer *et al* 1990).

The enzymic activity responsible for the conjugation of leukotriene A_4 with GSH to produce leukotriene C_4; and leukotriene A_4 methyl ester with GSH to produce leukotriene C_4 methyl ester was found to be associated with the microsomes (Yoshimoto *et al* 1985) Initially it was thought that the microsomal GST might be responsible however, a separate enzyme was purified to homogeneity from microsomes to account for this activity (Yoshimoto *et al* 1988).

In protection against oxidative stress there are two points at which the peroxidative process can be terminated. Firstly, species that initiate peroxidation such as free radical generators or free radicals themselves can be removed. Secondly, the removal of products of peroxidation, which further propagate the process such as fatty acid hydroperoxides is required.

As mentioned earlier there are a number of enzymes capable of carrying out these processes; glutathione-dependent enzymes are of pivotal importance. There are a number of glutathione-dependent enzymes which possess peroxidase activity and can carry out the reaction below:-

$$ROOH + 2\ GSH \text{ ----------> } GSSG + ROH + H_2O$$

One such enzyme is the glutathione peroxidase, which is a cytosolic selenoenzyme. Substrates are hydrogen peroxide, organic peroxides, free fatty lipid hydroperoxides and removal of these compounds inhibits lipid peroxidation *in vitro* (McCay, 1976). The alpha class GSTs, known as the selenium-independent glutathione peroxidases have glutathione peroxidase activity towards free fatty acid hydroperoxides and organic hydroperoxides, but not hydrogen peroxide (Tan *et al* 1984).

Lipid peroxidation can be a problem for the cell. Many macromolecules can be attacked creating a vast diversity of peroxidative products. The GSTs with their diverse substrate capacity and varying

Table 1.3 The specific activities for glutathione dependent enzymes towards substrates related to lipid peroxidation.

	Glutathione-dependent enzyme activity (μmol/min/mg).												
	[a]Rat cytosolic glutathione S-transferases.								[b]rat mGST.		Glutathione peroxidases.		
Substrates.	1-1	2-2	3-3	4-4	5-5	6-6	7-7	8-8	unact.	act.	[c]PHGPx.	[d]mGPX. +LPx.	
Lineolic HOOH.	3.0	1.6	0.2	0.2	5.3	-	1.5	-	0.2	0.6	0.34	0	71.2
4 HNE.	2.6	0.7	2.7	6.9	-	-	1.4	170	0.5	-	-	-	-
Cholesterol 5,6oxide.	10^{-3}	-	0	0	-	-	-	-	10^{-5}	-	-	-	-
DNA HOOH.	0	-	0.02	0.03	3.5	-	0.01	-	-	-	-	-	-
Cumene HOOH.	1.4	3.0	0.1	0.4	12.5	-	0.01	1.1	0.08	0.8	3.8	20.3	19.4
H_2O_2.	-	-	-	-	-	-	-	-	<.02	<.02	-	13.1	7.2
Phoshatidyl choline dilinoleic acid HOOH.	-	-	-	-	-	-	-	-	0.3	0.9	2.2	1.1	43.2
t-butyl HOOH.	-	-	-	-	-	-	-	-	-	-	0.9	0	1.5

References:
[a]Ketterer *et al* (1990) In: GSTs and drug resistance. 97-109.
[b]Mosialou and Morgenstern (1989) Arch.Biochem.Biophys. **275** 289-294.
[c]Ursini *et al* (1982) Biophys. Biochem. Acta **710** 197-211.
[d]Duan *et al* (1988) J.Biol. Chem. **263** 19003-19008.

Abbreviations:
mGST: microsomal GST; unact: unactivated; act:NEM activated; PHGPx: phospholipid hydroperoxide glutathione peroxidase; mGPx: monomeric glutathione peroxidase; LPx: lubrol-PX; Se-dep. GPx: selenium-dependent glutathione peroxidase; HOOH: hydroperoxide; 4HNE: 4-hydroxy-2,3- *trans*-nonenal; H_2O_2: hydrogen peroxide.

activities may detoxify many of these products within their metabolism (see Table 1.3). DNA peroxides, which are also formed during oxidative insult, are metabolised by the mu class GST 3 and 4. The lipid peroxidation products such as 4-hydroxy-non-2-enal, a hydroxyalkenal, are also toxic to cells. The alpha class enzyme 8-8 has particularly high activity towards these compounds (Ketterer and Meyer 1989). In assessing any one particular enzymes role in this process; the relative amounts, specific activities, tissue and species differences, all need to be considered.

Both the selenium-dependent and independent peroxidases can only metabolise fatty acid peroxides once they have been cleaved from the glycerol moiety by phospholipases (McCay *et al* 1976; Tan *et al* 1984). Indeed, glutathione peroxidase activity can be reduced by the addition of phospholipase A_2 inhibitors ρ-bromophenacyl bromide and mepacrine (Sevanian *et al* 1983). The activity of phospholipase A_2 is indirectly regulated by calcium, which is released under oxidative stress conditions. It is thought that the positive charge of the calcium ion interacts with the negative residues of the fatty acid hydroperoxides rendering them more accessible to the phospholipase A_2. The packing density, mobility and "flip-flop" are all altered when lipids are oxidised, which also makes them a better substrates for phospholipase A_2, than the undamaged form (Sevanian *et al* 1983).

The microsomal GST was shown to have glutathione peroxidase activity towards cumene hydroperoxide, which was co-purified to homogeneity with the protein. The enzyme, purified in its unactivated form, was shown to have activity towards cumene hydroperoxide that could be stimulated upon treatment with NEM (Reddy *et al* 1980).

Due to the cellular location of the microsomal GST it is an ideal candidate to afford protection to membranes from peroxidation. The free fatty acids when released from the membrane are still hydrophobic in nature and remain associated with the membrane. Mosialou and Morgenstern (1989) demonstrated that lipid peroxides were substrates for the microsomal GST and when the enzyme was activated the activity was comparable with certain cytosolic GSTs. As can be seen from Table 1.3, the microsomal rates are more comparable with the lower activities. However, the higher local concentration affect achieved by the membranes should be taken into

consideration when assessing the ability of the microsomal GST to metabolise these substrates. There are other factors which apply specifically to the microsomal GST such as, 4-hydroxy-2,3-*trans*-nonenal (4HNE), a hydroxyalkenal, which is a substrate for the microsomal GST (Mosialou and Morgenstern 1989) (although low again compared with the cytosolic GST, Table 1.3), but can also activate the enzyme via covalent modification (Haenen *et al* 1987). The cellular changes that occur during oxidative stress can increase the activity of this enzyme, were discussed in the last section.

Unlike the cytosolic enzymes, it has been suggested the microsomal GST could act on phospholipid hydroperoxides prior to phosphilipase cleavage, however no direct evidence was ever supplied by the authors (Mosialou and Morgenstern 1989). Two glutathione peroxidases, distinct from the classical selenium dependent form have been isolated, which have such activity towards uncleaved phospholipid hydroperoxide (Ursini *et al* 1982; Duan *et al* 1988), but this activity is somewhat enhanced by the presence of detergent. The phospholipid glutathione peroxidase has a synergistic relationship with the free radical scavenger α tocopherol and is closely associated with membranes although it is a truly cytosolic protein (Ursini *et al* 1982).

Glutathione itself can also act directly as a scavenger. Before the onset of lipid peroxidation in microsomes there is a glutathione-dependent lag phase (Burk 1983). The length of the lag was shown to be directly proportional to the concentration of glutathione, but the rate of peroxidation that ensued was unaffected. The lag could be abolished by heat suggesting a protein in the microsomes to be responsible. This protein is thought to act by free radical scavenging preventing the onset of peroxidation. The length of the lag is affected by the α tocopherol content, although a distinct but small effect was observed in the absence of α tocopherol. Hence, this protein may also act to reduce vitamin E radical produced during peroxidation - a vitamin free radical reductase.

The microsomal GST has been suggested as a candidate for the free radical reductase. This was based on the fact that inhibitors of the GST, rosebengal, tributyltin, 8-hexyl GSH, indomethacin, cibracon blue and bromosulphothalein could remove the lag period without affecting the

α topocherol content (Yonaha *et al* 1987; Mosialou and Morgenstern 1989). These are all general inhibitors of glutathione-dependent enzymes, however specific characteristics of the microsomal GST, such as covalent modification with NEM and 4HNE did not occur, in fact both these two compounds reduce the GSH-dependent lag while increasing the microsomal GST activity (Guido *et al* 1987; 1988). Additional conflicting evidence was presented by Morgenstern *et al* (1990) to fuel the debate in the literature, these studies showed that NEM did not affect the lag when fresh microsomes and 5mM GSH were used. Cystamine was used to replace NEM, as being more hydrophilic in nature and would have limited access to the membrane, thereby not damage the antioxidant properties of such thiols. Under these conditions, microsomal GST activity was increased and the lag phase extended.

The studies with allyl alcohol, which is metabolised to acrolein, argue for the existence of two separate proteins (Haenen *et al* 1988). *In vitro* acrolein activates the microsomal GST and inhibits the free radical reductase. *In vivo* administration to rats activates the microsomal GST, but the free radical reductase is unaffected. This certainly suggests this sulphydryl group on the microsomal GST more accessible to the solvent phase, but does not suggest the radical reductase is the microsomal GST. The free radical reductase has not as yet been purified or definitely assigned to a protein. Glutathione has also been shown to mediate free radical scavenging via a NADPH-dependent mechanism (Packer *et al* 1989). All these mechanisms are located in the microsomes, however the relative contribution of each in the protection against oxidative stress has not been determined.

Some evidence exists to suggest that the microsomal GST is in fact inhibited by lipid peroxidation. Treatment of microsomes with phosphyatidyl choline vesicles could activate the enzyme. Folch extraction of these vesicles after treatment found the inhibitory substance to be in the organic phase, it was suggested to be peroxidised lipids (Boyer *et al* 1982). Treatment with bovine serum fraction V powder had a similar effect, which was attributed to the property of bovine serum albumin to bind hydrophobic compounds lipids among them (Nishino 1989). In both cases the effect of NEM was additive suggesting a different mechanism.

A semi *in vitro* study was performed to show that lipid peroxidation inhibited the microsomal GST activity (Harris and Stone 1988). The study describes the effect of lipid peroxidation on microsomal GST activity from microsomes prepared from rats under two different diet regimes. Rats were fed on a control diet and one that would potentate lipid peroxidation, ie., deficient in selenium and vitamin E. When microsomes from the deficient-diet rats were challenged with a lipid peroxidation system, transferase activity was observed to fall. In the control system, the activity was sustained if not slightly raised, then fell in a similar manner to the microsomes feed on the control diet. However, the microsomal GST activity from the deficient diet was significantly higher than those from the control diet (Harris and Stone 1988).

Lipid peroxidation is a complex process affecting many areas of the cell. The literature is full of debate on the contribution of individual enzymes and the relative importance of various changes. When reviewing the literature for the microsomal GST, this case is no exception. However, the microsomal GST could potentially be regulated by this process and also can metabolise the products of peroxidation. The full impact of its contribution to the entire cellular physiology has not yet been fully addressed.

1.6 The involvement of the microsomal glutathione S-transferase in the formation of toxic products by glutathione conjugation.

In comparing the microsomal GST with the cytosolic enzymes it appears that in the majority of cases the cytosolic forms have higher affinities and activities for putative substrates. This has been the case for substrates such as lipid hydroperoxides, CDNB and hydroxyalkanels. Any contribution made by the microsomal GST, when these factors are considered, may be negligible.

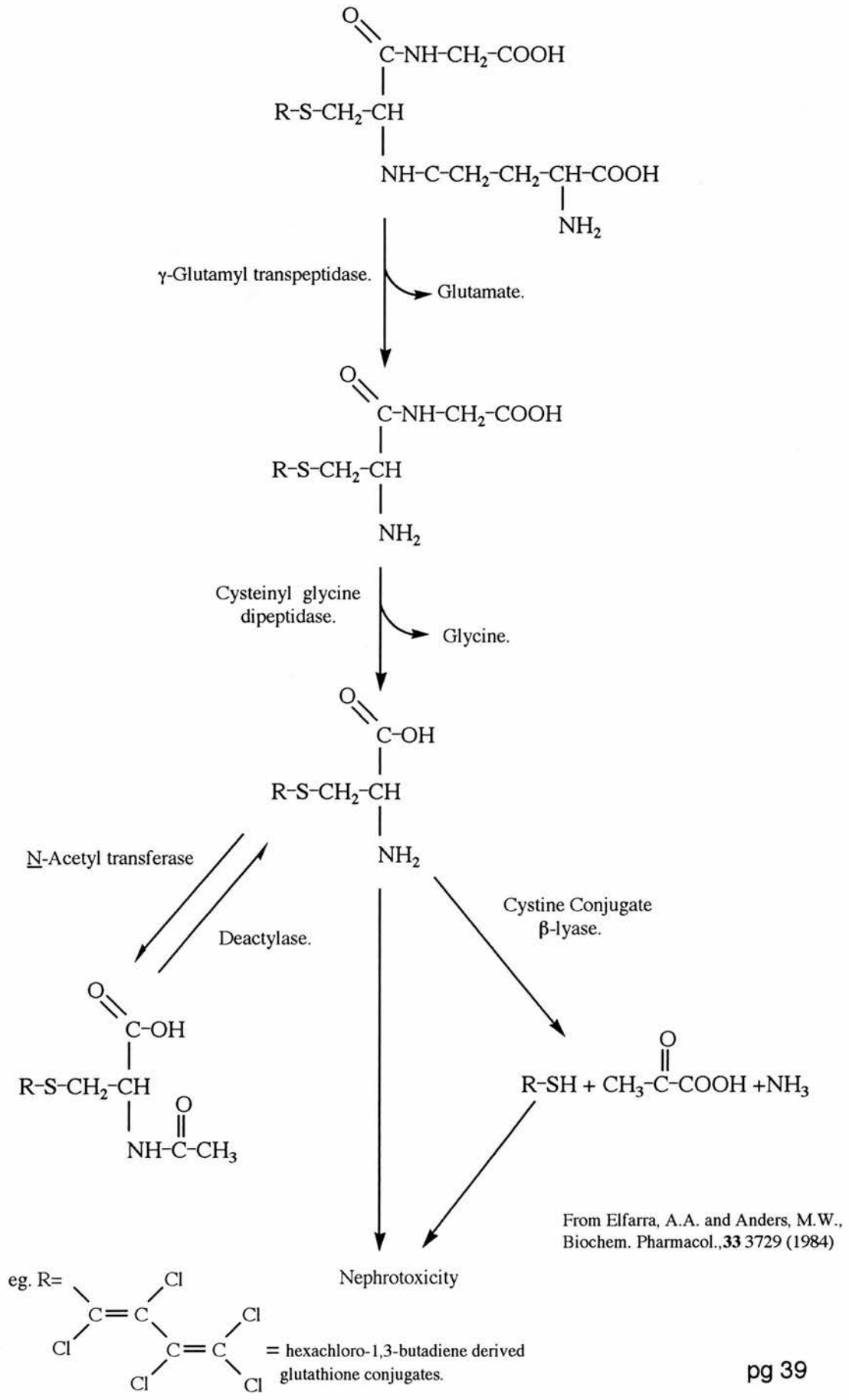
There are several classes of compounds which are converted by glutathione conjugation to toxic metabolites and in some cases the microsomal GST seems to be the predominant transferase in mediating these reactions. The types of compounds involved are halogenated hydrocarbons and are often byproducts of industry, hence their detailed study.

The simplest examples are the vicinal dihaloalkanes, such as 1,2-dibromoethane and 1,2-dichloroethane. The P450 system plays the predominant role in the metabolism of these compounds (Van Bladeren *et al* 1981). There are a number of reactive species produced that are thought to be responsible for covalent binding to proteins and observed cytotoxicity (Guengerich *et al* 1980). In isolated microsomes a synergistic relationship exists between NADPH and glutathione-dependent metabolism. The addition of glutathione increases binding to proteins, but P450 metabolism is a prerequisite (Guengerich *et al* 1980).

Direct glutathione conjugation leads to the formation of the highly reactive half mustards S-(-2 bromoethyl) glutathione and S-(-2 chloroethyl) glutathione (Marchand and Reed 1989). These are particularly strong alkylating agents and bind to DNA inducing a genotoxic response (Van Bladeren *et al* 1980). The major adduct formed is 2-(N^7guanyl)ethyl glutathione (Ozawa and Guengerich 1983). Investigation of the reactivity of individual glutathione S-transferases towards 1,2-dibromoethane showed the alpha class (rat, 2-2 and human B_1B_1 and B_1B_2) to have significantly higher activity than other classes. The microsomal GST had only limited ability to directly conjugate this compound (Camarik *et al* 1991). However, no measurements were made with activated enzyme or P450 metabolites, which may be relevant in this case (Botti *et al* 1982; Lundqvist and Morgenstern 1992b).

Assessing the relative contribution of the various glutathione transferases to the metabolism of any one compound is difficult. In some cases stereoselective glutathione conjugation with substrates has been observed producing products with different stereochemistry depending on the enzymes involved (te Kopple *et al* 1991). In terms of catalytic mechanism this may be explained by subtle differences in he G site and the precise orientation at which the thiolation is present for catalysis (Adang *et al* 1990). Chlorotrifluorethene is an example of such a prochiral substrate, in which conjugation results in the formation of a new chiral centre. An excess of one diasteromer is produced by the microsomal GST, but equal amounts with cytosolic transferase (Dohn *et al* 1985). Using this information the overall metabolism of this compound in the hepatocyte can be investigated and the majority of the conjugate produced was attributed to the microsomal

Figure 1.5 Metabolism of S-substituted glutathione conjugates.



From Elfarra, A.A. and Anders, M.W., Biochem. Pharmacol.,**33** 3729 (1984)

GST (Hargus *et al* 1991).

The chlorinated alkene, hexachlorobutadiene (HCBD) is one of the best characterised substrates for the microsomal GST (Wolf *et al* 1984). The specific activity of the microsomal enzyme is 3-4 times higher than the cytosolic enzymes (Oesch and Wolf 1989). The lipophilic nature of these compounds is a common feature, which results in preferential distribution into the membrane causing a higher local concentration. When this factor is taken into account the microsomal GST is reported to have a 116 times higher activity towards HCBD (Wallin *et al* 1988). Compounds such as HCBD, trichloroethane, tetrachloroethene and dichloroacetylene are exclusively metabolised by the GST and not the P450s (Wallin *et al* 1988). However, it is not the glutathione conjugate that is responsible for the toxicity, but the metabolites of the mercapturic acid pathway along which the conjugate travels (see Figure.1.5 adapted from Elfarra *et al* 1984).

The importance of each step has been characterised by studies using inhibitors and substrate analogues of the enzymes involved. The major site of glutathione conjugation is the liver, then the conjugate is then transported in the blood to the kidney and is taken up across the brush boarder membrane where the glutamyl moiety is removed by γ glutamyl transferase (γGT). Modification by cysteinylglycine dipeptidase yields the S-cysteine conjugate, which is metabolised by the mitochondrial β lyase. The products of this reaction are ammonia, pyruvate and thiol compounds. It is the thiol compounds which are highly reactive and are thought to mediate the toxic effect. Other enzymes such as γlyase can produce similar toxic compounds by γ eliminations (Anders *et al* 1988). The kidney is the major site of such metabolism and it is here that the toxic effects are observed.

The main cellular site for toxic insult by these compounds are the mitochondria, this is interesting in terms of the similarities to the toxicity elicited by oxidative stress. Cellular respiration is inhibited in the case of S-(1,2-dichlorovinyl)-L-cysteine (the S-cysteine conjugate of HCBD). The point of inhibition appears to be at complex II and results in a loss of ATP (Wallin *et al* 1987). Inhibition of the TCA cycle enzymes succinate cytochrome c oxidoreductae and isocitrate dehydrogenase is observed. These enzymes require free sulphydryl groups for activity, and it is the thiol

metabolites produced by the β-lyase that react with critical thiols in these proteins (Lash and Anders 1987). Similar to the observations made during oxidative stress, membrane potential collapses, calcium ions are released and disruption to anion and cation transport are seen (Anders *et al* 1988).

Reactive thiol species might be expected to activate the microsomal GST, but this has not been reported. However, when HCBD was used as a substrate the enzyme could not be activated by NEM or trypsinisation (Oesch and Wolf 1989). A possible explanation may be the enzyme had already been modified by HCBD metabolites. The Vmax value given by (Wallin *et al* 1988) falls within the range in which substrates are activatable (Morgenstern *et al* 1988). However, the values reported by Oesch and Wolf (1989) are not, which may also explain their observations.

As might be expected from such observed modes of toxicity antioxidants can protect against S-conjugate induced toxicity without inhibiting covalent binding to mitochondria (Chen *et al* 1990). Significantly, in this respect, the amount of [S^{35}] from labelled GSH bound to macromolecules does not relate to the toxicity observed, indicating that thiol loss is not the only toxic effect mediated by these covalent modifications. In this respect Ca^{2+} chelators can also prevent toxicity and inhibit membrane blebs. It has been suggested that blebbing is caused by the Ca^{2+} and Mg^{2+}-dependent phospholipases activated during the calcium release from mitochondria (Koob and Dekant 1990).

HCBD also produces a mutagenic effect in the Ames test. However, exogenous β-lyase and γGT maybe required to see an effect depending on the strain of *Salmonella* (Vamvakas *et al* 1988). The toxicity and carcinogenicity is confined largely to the kidney in rodents. In a mammalian cell model LLC PKI cells (a porcine kidney cell line) it has been shown that glutathione S-conjugates can cause DNA damage. This was judged by an increase in unscheduled DNA synthesis, but this was somewhat lower than other DNA damaging agents. DNA binding was largely observed in the mitochondria highlighting this as the primary organelle for attack (Vamvakas *et al* 1989).

The conditions found in the cell during this form of toxic insult are similar to those found during oxidative stress and are suitable for modulating the microsomal GST activity, but in this case the action of the enzyme

appears to potentate toxicity.

1.7 Summary and aims of the thesis.

The microsomal GST is somewhat of an enigma. The protective role of glutathione conjugation within cell has long been studied, but does this enzyme fall into the same category as the cytosolic GSTs in this respect? The differences between the microsomal and cytosolic enzymes have been outlined in this chapter and are highlighted by the lack of sequence homology, lower turnover numbers and different regulatory mechanisms. Such radical differences coupled with the lack of evolutionary relationship perhaps suggests that the microsomal GST has a different function within the cell

This review has focused on the protein modifications that alter the enzyme activity as a means of understanding the physiological role of this particular enzyme. The protein is located in the hydrophobic environment of a number of membranes and it possesses a single cysteine residue which is susceptible to covalent modification by reactive intermediates and oxidised thiol compounds. The covalent modification seems to result in a confirmational change that allows the binding energy of glutathione to be used more efficiently. There has been suggestion that in doing so the catalysis is driven towards a more cytosolic GST-like nature.

To understand the function of the microsomal GST the conditions under which activation of the enzyme occurs have been examined. Reactive species produced during xenobiotic metabolism and conditions under which thiol status in the cell was disrupted cause activation of the enzyme. Naturally, in a number of cases the two conditions are related, but the generation of species that can alkylate the thiol residue within the enzyme is the common denominator. Membrane damage is critical in the toxicity of a compound to a cell. A membrane enzyme with a latent activity activated by harmful agents could detoxify such compounds, or their products, and could therefore offer protection to membrane integrity.

The effect of upregulation of expression of the GSTs is thought offer increased resistance of the cell to toxic insult. For example, the cytosolic GSTs were shown to be raised in tumours and cell lines made resistant to

anti-cancer drugs (reviewed by Hayes and Wolf 1988). To test whether this over-expression could explain the observed phenotype these enzymes have been expressed in heterologus systems and the resulting model treated with the appropriate agents.

The microsomal GST has not been shown to be induced by xenobiotics at the transcriptional level like the cytosolic enzymes. However, placement of this enzyme in an expression system would allow the assessment of the contribution of the microsomal GST to exposure of the cell to a chosen agent. The DNA technology is available to allow the alteration of specific residues in a protein, by site directed mutagenesis (SDM). The opportunity to apply this technique to the microsomal GST has the enormous potential to yield information not only about the catalytic mechanism of the enzyme but the activation process as well. This thesis describes the expression of the microsomal GST in *Saccharomyces cerevisiae* and *Escherichia coli*. The yeast expression was then used to asses the role of the microsomal GST in the metabolism of a number of putative substrates.

Information about the expression of an enzyme within the organism can provide information about the role of an enzyme and the importance of such considerations have also been discussed. Studies were undertaken to investigate of tissue distribution and hormonal regulation in rodent species.

Chapter 2: Materials and methods.

2.1. Microbial Requirements.

2.1.1 Bacterial strains.

Escherichia coli (*E.Coli*) DHI (F-*recA1, gyrA* 96,*thi* 1,*hsd* R17,*sup* E44)
E.Coli JM109 *e14* (*mcr A*), *rec A1, end A1, gyr A96, thi-1.*
E.Coli NM522 *Sup E thi-1* D(*lac-pro AB*), D(*hsd SM M-crB*)5 (n_K-m_K)
-these strains were used as they are *rec-* and are suitable for large scale growth and plasmid preparation.
E.Coli JM 101 dam-*Sup E, thi-1,* D(*lac-pro AB*), [F', *traD 36, pro AB, lac* I^q*Z* D *M15*] used where unmethylated DNA was required eg when a *Cla* 1 site was being employed eg Section 5.40.
E.Coli. RM82 containing PUBS 520, a gift from Professor R. Mattes, Boeringer Mannheim.
E.Coli. TG-1 containing the F pili required for single strand DNA preparation of M13 phage.
E.Coli. BL21(DE3) pLysS containing T7 RNA polymerase and a T7 lysozyme on a plasmid under chloramphenicol selection under laq I^q control.

2.1.2 Bacterial culture media.

L-Broth/litre: 10g bacto-tryptone (Difco);5g Yeast extract (Difco); 5g sodium chloride; 1g glucose (BDH).L-Agar contains in addition 15g of agar per litre.
2xTY/litre:16g Bacto-tryptone;10g Yeast extract; 5g sodium chloride.
Terrific broth/litre: 24g yeast extract;12g bacto-tryptone;4ml glycerol, 100ml 0.17M potassium dihydrogen orthoposphate, 0.72M di- potassium hydrogen orthoposphate.
H-bottom: 10g bacto-tryptone;8g sodium chloride.H-bottom Agar contains 15g agar/litre.H-top Agarose contains 6g agarose per litre.

2.1.3 Antibiotics.

Antibiotic.	Stock conc.	Working conc.
Ampicillin- Amp(Beechams-penbrit).	50mg/ml(H_20).	50μg/ml.
Kanomycin-Kano(Sigma).	10mg/ml(H_20).	50μg/ml.
Chloramphenicol-Chloro(Sigma).	34mg/ml(ethanol)	10μg/ml.

These were added as required to the appropriate media depending on the plasmid under selection.

2.1.4 Bacterial Plasmids.

Plasmid	Function.	Selection.
pUC 18/19	cloning	Amp^r
pJM9	expression	Amp^r
OmpA	expression	Amp^r
pET 3a and pET 3	expression	Amp^r
pGEX-2T	expression	Amp^r
pTZ 18/19	cloning	Amp^r
M13mp18/19	sequencing	
pUBS520	expression t^{RNA}_{arg}	$Kano^r$
pAX 11	expression	$Kano^r$
pHL 1	expression	Amp^r

2.1.5 Bacterial transformation using calcium chloride.

Competent *E.Coli* cells were prepared essentially by the method described by Maniatis *et al* (1982). An overnight culture was diluted 1:100 in LB and grown for a further two and a half hours, until the absorbence was at 600nm was 0.5. The cells were then pelleted and resuspended in a fifth of the culture volume of 50mM calcium chloride ($CaCl_2$) and incubated for 20 min. on ice. The cells were again pelleted, but resuspended in a tenth of the culture volume of 50mM $CaCl_2$ and left on ice for a further hour.

The ligation mix or plasmid to be transformed (10ng-1mg DNA) was added to a 100μl aliquot of cells and left for half an hour on ice, and then the cells were heat shocked at 42°C for 5 minutes. After pre-expressing the cells

for an hour in liquid culture at 37°C, the cells were plated onto LB agar containing the appropriate selectable antibiotic and incubated overnight at 37°C.

2.1.6 Transformation of bacteria with M13 phage.

Competent TG-1 *E.Coli* strains were prepared as described above, only the cells were grown in 2xTY as opposed to LB in order to maintain the F pili episome required for transfection with M13.

The M13/insert ligation mix was added to 200μl competent cells and left on ice for one hour, and then heat shocked at 42°C for 2 min. Sterile culture tubes were prepared at 55°C , containing 3ml of molten top agarose, 40μl of 100mM isopropyl-β-D-thiogalactopyranoside (IPTG), 40μl 2% 5-bromo-4-chloro-3-indocyl-β-D-galactoside (XGal) and 200ml of TG-1 in stationary phase. The 200μl of competent cells mixed with DNA were added to the culture tubes and immediately plated out. After the top agarose had set the plates were inverted and incubated at 37°C overnight. The plaques which were transfected with M13 containing insert were clear where as M13 alone transformed cells were blue.

2.1.7. Yeast strains.

Saccharomyces Cerevisiae. KY118 (a,his3-200,lys2-801^{am}ade2-101^{oc},trpD1, ura3-52), a gift from Dr.J.Beggs, Dept. Molecular Biology, Edinburgh University.

Saccharomyces Cerevisiae .W3031B (a,leu2,his3,trp1,ura3,ade2-1,can^{R},cyr^{+}), a gift from Dr.D.Pompon,Centre de Genetic Molecular du CNRS,Laboratoire Propre associe a l'Universite Pierre et Marie Currie, 91190 Gif-sur-Yvette, France.

2.1.8 Yeast Culture media.

YPD/litre:10g bacto-peptone(Difco); 10g yeast extract; 20g glucose; 20mg adenine sulphate(Sigma). Adenine sulphate is only required for

adenine minus strains, although was always included. YPD-Agar (YPDA) contains 20g per litre agar.

Yeast Minimal Media (YMM): 6.7g yeast nitrogen base without amino acids (Difco); 20g Glucose; 20mg amino acids and vitamins to give supplemented synthetic medium SD as required.

Supplemented synthetic SD medium plus cas amino acids (YMM+Cas): YMM with the addition of 10g Cas amino acids (1%) per litre.

S6 0.7% yeast nitrogen base, 5% glucose (for induction of the PGK promoter), 0.1% Cas amino acids (or 1% or S6+Cas), 20 mg tryptophan, 40 mg adenine, per litre (Cullin *et al* 1988).

S5 same as S6 but 5% glucose was replaced with 2% galactose for induction of the Gal10 cyc1 promoter.

2.1.9 Yeast Plasmids.

Plasmid.	Promoter.	Selection.	Reference.
pMA 56	ADC1	trp	Ammerer *et al* (1983)
pYEDP1/8-2	Gal10cyc1	ura	Cullin *et al* (1988)
pYEDP1/10-1	PGK	ura	Cullin *et al* (1988)
pVT 100U	ADH	ura	Vernet *et al* (1987)
YEplac 112	-	trp	Gietz *et al* (1988).

2.1.10 Transformation of yeast using the lithium acetate method.

Yeast were transformed using the lithium acetate method according to Itoh *et al* (1983). This involved treating a early Log. phase culture (OD_{600nm}=0.4) suspended in TE buffer (10mM Tris pH 8.0 and 1mM EDTA) with lithium acetate (final concentration 100mM). The DNA was then added and after incubation for half an hour, poly ethylene glycol (PEG) was added at a final concentration of 35% in order to facilitate the transfer of DNA through the yeast cell wall. After heat shock, 5 minutes at 42^{0}C, the PEG was removed and the cells resuspended in distilled water. The cells were plated out on a YMM agar with a suitable cocktail of amino acids to allow the selectable marker on the vector to complement the deficiency. The plates were incubated at 28^{0}C until colonies appeared.

2.2. DNA techniques.

2.2.1 Plasmid preparations.

2.2.1a Alkaline lysis (Brinboin and Doly 1979).

The method was adjusted according to the size of culture used. The pellet was resuspended in 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA, 2mg/ml lysozyme(Sigma) and after 30 minutes 1%SDS and 0.2M NaOH were added to disrupt the cells. The bacterial genomic DNA and cell debris were precipitated by the addition of 3M sodium acetate (NaOAc). After a 30 minute incubation on ice, the precipitate was removed by centrifugation and the plasmid DNA was precipitated with isopropanol. The plasmid was further purified by a phenol/chloroform extraction, which was followed by ethanol precipitation, or for large cultures by density gradient centrifugation through caesium chloride (Boeringer-Mannheim). The purified plasmids were resuspended in TE and stored at -20^{0}C. The presence of the plasmid was determined by analysis on an agarose gel. For constructs, a suitable restriction digest was carried out to determine the presence of the insert and its orientation within the vector.

2.2.1b Quiagen columns.

Quiagen columns is the trade name of the product used for rapid purification of high quality plasmid DNA. Essentially, the basic procedure is as above, but carried out with the manufacturers buffers. After the removal of the bacterial genomic DNA the solution is passed over a DNA affinity resin to which the plasmid DNA binds. This is then eluted with a high salt buffer and isopropanol precipitated in the final step.

2.2.2 Restriction endonuclease digest.

Restriction enzymes were purchased from Boeringer-Mannheim, New England Biolabs, BRL and Amersham International. The reaction mixture was set up according to the manufacturers instructions and according to the

requirements of the individual enzyme. After digestion the reaction was stopped with a tenth volume of 20% ficoll, 10mM EDTA and bromophenol blue loading dye and analysed by agarose gel electrophoresis. However, if the products were required for a subcloning step, the reaction mixture was phenol/chloroform extracted followed by ethanol precipitation and resuspended in the appropriate buffer for the next manipulation.

2.2.3 Gel electrophoresis of DNA.

Two running buffers were generally used and these were TBE (89mM boric acid, 89mM Tris, 2mM EDTA) and TAE (0.4mM Tris pH 8.2, 0.2M NaAc and 10mM EDTA). The gel was composed of the appropriate running buffer and 1% agarose. A higher concentration of agarose was used for the resolution of smaller fragments. After electrophoresis the gels were stained in ethidium bromide and viewed on a UV transilluminator.

2.2.4 Isolation of cDNA fragments.

This procedure was carried out in order to facilitate subcloning and the fragments were isolated using the gene clean kit. The procedure was carried out according to the manufactures instructions and involved resolving the fragments on a low melting point TAE agarose gel, excising the band desired and removal of the agarose in sodium iodide. The cDNA was extracted by binding onto glass milk™ and finally elution into distilled water.

2.2.5 Ligation of fragments into vector.

The ligation of fragments was carried out using T_4 DNA ligase and according to the recommendations of the suppliers, Boeringer-Mannheim. The reaction took place overnight at room temperature.

2.2.6 Dephophorylation of DNA using calf intestinal alkaline phosphatase (CIAP).

The CIAP was supplied by Promega and the reaction was set up with

the desired cDNA or vector, according to the manufactures recommendations. After the reaction was complete, the reaction mixture was generally phenol/chloroform extracted in order to facilitate a buffer change for the next manipulation.

2.2.7 The polymerase chain reaction (PCR).

This technique was employed to engineer the rat microsomal cDNA to a form suitable for cloning into a range of expression vectors. For example, where a ribosomal binding site needed to be incorporated 5' of the ATG in a bacterial expression vector or a fusion in frame with another protein was required. The precise details of such examples are discussed at the appropriate point in the text.

For a 100μl reaction mix the following were added; 1μl of the DNA template (~1-3ng of DNA), 1μl of each oligo (1mg/ml), 8μl of dNTPs (2.5mM), 10μl of 10x Promega reaction buffer] (10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$. 0.01% gelatin) and 0.5μl of DNA Taq 1 DNA polymerase 4U/ml (Promega); the volume was made up to 100μl with distilled water and overlayed with mineral oil after mixing.

The Techne PCR machine was programed to carry out 30 cycles of PCR:

1). 5 minutes at 92^{0}C.

2) a. Melting: 92^{0}C. for 30 seconds

b. Annealing:50^{0}C. for 45 seconds

c. Extension: 72^{0}C. for 60 seconds

3). 5 minutes at 72^{0}C.

Section 2) was repeated 30 times and was responsible for the amplification of the DNA. The efficiency of the reaction was assessed by a 10μl aliquot being run on an agarose gel. The fragments were purified using gene clean (Section 2.24), before exposure of the restriction enzyme sites using a restriction digest.

2.2.8 The random priming of isolated cDNA with radiolabelled ^{32}P α dCTP.

The cDNA to be labelled was isolated from low melting point agarose, 1.5 ml of distilled water was added to every gram of agarose and heated to 100^{0}C. for 10 min., after which a 20ml aliquot was withdrawn for labelling and the remainder store at -20^{0}C.

The molten agarose containing the DNA was added to 5μl OLB (oligo labelling buffer, as described in Maniatis *et al* (1982), 2μl BSA (bovine serum albumen), 18μl of water, a ^{32}P dCTP (final activity 30mCi) and 2μl of Klenow (Klenow fragment of *E.Coli* DNA polymerase I).

The labelling reaction was carried out overnight at room temperature or for two hours at 37^{0}C. The amount of label incorporated was assessed by chromatographic separation of unincorporated labelled nucleotides from labelled DNA, using a 2μl aliquot on DEAE 81 paper in 0.4 ammonium formate pH 8.0, followed by exposure to photographic film. Before using the probe the reaction mixture was heated to 100^{0}C for 5 minutes to melt the DNA.

2.2.9 Klenow reaction to fill in cohesive ends of DNA.

The method for end filling of DNA is essentially described in Maniatis *et al* (1982). Great care was taken to removed any restriction enzyme which might be present. The plasmid was resuspended in a total volume of 50μl, which contained TM buffer (10mM Tris-HCl pH 8.0, 1.5mM $MgCl_2$), 0.25mM dNTPs, and 1μl of Klenow fragment. The reaction was carried out for 30 min. at 37^{0}C. followed by a phenol/chloroform extraction.

2.2.10 Screening bacteria for the presence of recombinant plasmids.

The method of Grunstein and Hogness (1975) was used to screen large numbers of bacteria for transfectants which contained plasmid with the

desired insert. The method involves streaking out individual colonies in duplicate onto nitrocellulose filters (Schleicher and Schuell) which had been previously placed on antibiotic selectable plates. Following incubation over night at 37^{0}C, one plate was stored at 4^{0}C, while the other filter was subjected to 10% SDS, denaturation (0.5M NaCl, 1.5M NaOH) and finally neutralisation (0.5M Tris-HCl pH 7.5, 1.5M NaCl). This caused the bacteria to be lysed and release their DNA, which was adhered to the filters by baking for 1 hour at 80^{0}C. The filters were washed to remove bacterial debris in 50mM Tris-HCl pH 8.0, 1.5M NaCl, 1mM EDTA and 1% SDS at 42^{0}C. Filters were then incubated at 65^{0}C for two hours in 6xSSC(1xSSC= 150mM NaCl and 15mM sodium citrate) 2xDenharts (1x Denharts= 1mg/ml of ficoll,PVP* and BSA), 0.1% SDS and 0.1% sodium pyrophosphate, after which the radio labelled probe, corresponding to the prospective insert, was added. After overnight hybridisation, the filters were washed in 2xSSC and 0.1% SDS at room temperature followed by several washes in 1xSSC and 0.1%SDS at 65^{0}C. The dried filters were exposed to autoradiography film and colonies in which the vector contained an insert gave a strong signal.

Alternatively, plasmid preparation was performed on a small sample (1.5ml) of a overnight culture of individual transformants. Not as many colonies could be screened as with the Grunstein and Hogness method.

2.1.11 Preparation of single-stranded templates for sequencing.

An overnight culture was inoculated at a dilution of 1:100, and then a 1.5 ml aliquot was placed in a sterile bijoux with a colourless plaque picked on the end of a sterile cocktail stick. The culture was then vigorously shaken at 37^{0}C for 5 hours, after which the cells were pelleted in an eppendorf. One ml of supernatant was removed and placed in a sterile eppendorf and the process was repeated in order to ensure that no cells were present. The final 1ml was added to 200μl of PEG/NaCl (20% PEG and 2.5M NaCl), which was then shaken on ice for one hour. The viral pellet was precipitated by centrifugation for 30 minutes and extreme care was taken to remove all traces of PEG/NaCl. The pellet was resuspended in 100μl of TE and subjected to a phenol/chloroform extraction and ethanol precipitated

overnight. The final pellet was resuspended in 10μl of TE, 7μl of which was used per sequencing reaction.

2.1.12 Single stranded sequencing of M13 derived clones using Sequenase version 2.

Sequenase version 2 (United States Biochemical) is essentially the method of Sanger *et al* (1977) and was carried out according to the Manufacturers instructions.

After completion of the sequencing reaction the samples were resolved on a 6% acrylamide, 7M urea (BRL ultra pure grade) and 1xTBE gel, which after electrophoresis was fixed in 10% acetic acid and 10% methanol and dried before exposing to autoradiography.

2.1.13 Spectrophotometric quantitation of DNA and RNA.

RNA and DNA concentrations were measured spectrophotometrically at 260nm and the concentrations calculated accordingly:

1 absobance unit at 260nm = 50mg/ml for double stranded DNA.

1 absobance unit at 260nm = 40mg/ml for single stranded DNA and RNA.

Measurement of the absorbency gave an indication of the protein present and the ratio of the OD_{260}/OD_{280} gave a measure of the purity of the the nucleic acid, which for pure samples was 1.8 for DNA samples and 2.0 for RNA.

2.3. RNA techniques.

2.3.1 Extraction of RNA from rodent tissues.

RNA extraction was carried out according to the method of Cox (1968), which employs the powerful protein denaturant guanidinium hydrochloride (GnHCl) to destroy the ribonuclease activity which is released on disruption of tissues. The method relies on the differential precipitation of

RNA and DNA at -20^{0}C followed by centrifugation at 10000g.

These preparations were carried out by Dr.C.J.Henderson as described in Section 3.33.

2.3.2 Extraction of RNA from yeast.

A 200ml culture of yeast were grown to the appropriate optical density and the cells collected by a 10 minute spin at 6000g. The cell pellet was washed once in TNE (50mM Tris-HCl pH 7.5, 5mM EDTA, 100mM NaCl) and resuspended in 4ml of TNE and approximately 0.3ml of acid washed glass beads (BDH 40mm mesh). One sample was treated at a time, 0.2ml of 20% SDS and 1ml of phenol was added and vortexed for 1 minute at maximum speed. The mixture was spun at 3000g and the aqueous upper layer was removed and extracted with phenol/chloroform (1:1), followed by a chloroform/isoamyl alcohol (24:1) extraction. After the extraction processes the aqueous solution was ethanol precipitated overnight at -20^{0}C. The RNA was pelleted by a 10 minute 10000g centrifugation step and resuspended in DEPC (diethyl pyrocarbonate) treated water (0.01%) and stored at -70^{0}C.

2.3.3 Extraction of RNA from bacteria.

The bacteria were grown to the appropriate optical density and received the designated treatments, such as IPTG induction. Five ml aliquot of the culture were spun at 3000g and the resulting cell pellet resuspended in half the volume of prewarmed media, to which 250μl of 10x lysis buffer (0.5M Tris-HCl pH 7.4, 20mM EDTA and 10% SDS) was added and the mixture was vortexed rapidly. After boiling for 2 minutes, 250μl of sodium acetate was added followed by 1 ml of water saturated phenol and 1ml of chloroform. After centrifugation at 3000g the aqueous layer was extracted and mixed with an equal volume of phenol/chloroform (1:1). After extraction 2 volumes of ethanol were added and the RNA was allowed to precipitate overnight in siliconised tubes. The RNA was pelleted by a 10000g 10 minute spin and then vacuum dried. The pellet was resuspended in 0.4ml of DNAase buffer (40mM Tris-HCl pH 7.4, 10mM NaCl, 6mM $MgCl_2$) in an

eppendorf. The DNA was removed by adding 6μl of RNAase free DNAase (Boeringer-Mannheim 30U/μl) and incubating at 37^{0}C for 20 minutes. Following a phenol/chloroform extraction and ethanol precipitation the RNA was resuspended in DEPC (diethyl pyrocarbonate) treated water and stored at -70^{0}C.

2.3.4 Northern blot analysis.

RNA was separated on 1.5% agarose gels made in MOPS buffer (0.2M MOPS, 50mM sodium acetate and 10mM EDTA pH 7.0) containing 18% formaldehyde. Between 10-15μg of RNA was added to 3 volumes of sample buffer (0.2M MOPS, 0.6% formaldehyde and 50% formamide) and heated at 55^{0}C for 30 min., then a tenth of the volume of loading dye (0.4% w/v xylene cyanol, 50% glycerol and 1mM EDTA) was added and the samples applied to the agarose gel. After electrophoresis RNA was transferred by capillary action to Hybond-N nylon filters (Amersham International) in 10x SSC. After overnight transfer the RNA was cross-linked to the filter using a Stratagene UV cross-linker. The filter was prehybridised in 6xSSC, 2x Denhardts, 0.1% SDS and 0.1% sodium pyrophosphate at 65^{o}C for 1-2 hours. After adding the probe the filter was hybridised overnight. The unbound probe was removed by washing 2-3 times in 2x SSC,0.1% SDS and 0.1% sodium pyrophosphate, after which the filter was dried and exposed to autoradiographic film.

2.4. Cellular preparation and analysis.

2.4.1 Preparation of yeast subcellular fractions.

This micro-organism has a particularly tough polysaccharide cell wall and as a result all procedures focus on means of breaking the cell wall. Three methods were used and are discussed below. The different procedures varied in the efficiency of cell disruption which was balanced against the heat generated due to the friction of the lysis process.

2.4.1a. Glass bead disruption of yeast.

Glass beads (BDH 40mm mesh) were washed in concentrated hydrochloric acid followed by repeated rinsing in distilled water until the pH had returned to neutral. The beads were then rinsed in the appropriate buffer and excess liquid removed with a pasttete.

The prepared beads were added to the cell suspension to approximately $^2/_3$ of the volume, then the mixture was vortexed at maximum speed for 2x2 min. and placed on ice in the interval. The resulting lysate was removed (using a Gilson) and stored at -40^{0}C. Such preparations were used largely for Western blot analysis, as the degree of breakage was low and samples were not suitable for subcellular fractionation or biochemical assays.

2.4.1b. Spheroplast method for disruption of yeast cells.

The culture was grown until an OD_{600nm}=0.6-0.8, because if grown for any longer the efficiency of digestion of the cell wall by Zymolase (ICN Flow laboratories) was greatly reduced.

The yeast cells were pelleted by a 5000g centrifugation, washed twice in distilled water and finally resuspended in 1.2M sorbitol (Sigma) and 50mM potassium phosphate buffer pH 7.5. Zymolase was added at a final concentration of 1mg/ml and incubated with gentle shaking for 30-45 minutes at 37^{0}C. The cells were assessed for spheroplast formation by removing a 10μl aliquot onto a slide and then adding water under the cover slip, cells which had formed spheroplasts turned rapidly dark and shrunk. The spheroplasts were washed very carefully in sorbitol buffer in an eppendorf using the low speed spin on the bench top centrifuge (6500rpm). Zymolase contains a number of contaminating proteases, which were removed by this washing process. After washing, the spheroplasts were resuspended in a hypotonic buffer, such as potassium phosphate/KCl buffer (10mM potassium phosphate buffer pH7.5, 0.1mM EDTA and 1.15% potassium chloride), and then briefly sonicated using a Soniprep 150 sonicator (MSE) with two 15 second bursts with cooling on ice between

bursts. Alternatively, the spheroplasts were lysed by gentle homogenisation in a glass-teflon homogeniser. The lysate was used immediately for biochemical analysis or subcellular fractionation.

2.4.1c. French Press for the disruption of yeast cells.

This method was more suitable for the treatment of cells harvested in late log. and stationary phase as the process could cope with thickened cell walls. Cells were harvested by a 5000g centrifugation step and washed in potassium phosphate/KCl, pH7.5 buffer before the final resuspension in two times the volume of the pellet. The suspension was then passed through the French press at a pressure of 200lb/inch^2. Care was taken to cool the sample chamber at 4^{0}C and collect the lysate on ice, however the process did generate a significant amount of heat. Once again the lysate was used immediately for biochemical analysis or subcellular fractionation.

2.4.2 Subcellular fractionation of lysed yeast cells.

This was based on the method of Yoshida *et al* (1974) and modified as described by Black *et al* (1990) and the various organelles were separated by differential centrifugation according to size.

The cell debris and unbroken cells were removed by a 6000g spin for 5 min., then the resulting supernatant was spun at 11000g for 20 minutes to pellet the mitochondria. Potassium phosphate/KCl, pH7.5 buffer plus 0.25M sucrose buffer was used to resuspend the mitochondria, after one wash in potassium phosphate/KCl, pH7.5 buffer. The 11000g supernatant was spun at 100 000g for 80 min., the supernatant was designated the cytosolic fraction and the pellet the microsomal fraction. The resulting pellet was washed three times in potassium phosphate/KCl, pH7.5 buffer by repeating the 100 000g spin for 60 min., before resuspending in potassium phosphate/KCl buffer plus 0.25M sucrose. The extensive washing of the microsomal fraction was designed to remove contaminating cytosolic proteins, especially the cytosolic GSTs which would interfere with the assessment of microsomal GST activity.

The exact organelle composition of each of the fractions was

assessed by Western blot analysis using antibodies to marker proteins; yeast alcohol dehydrogenase 1 for cytosol and yeast 14KDa outer mitochondrial membrane (14KDa OMM, Riezman *et al* 1983) for mitochondria. The microsomal fraction was assessed using the assay for the endoplasmic reticulum protein P450 reductase. The findings are discussed in Section 4.33.

2.4.3 Disruption of bacterial cells.

The bacteria were harvested by a 5000g spin and resuspended in an appropriate buffer for subsequent steps, then lysozyme (Sigma) was added at a final concentration of 1mg/ml and the suspension left on ice for 20 minutes. The cells were then sonicated for 2x15 and 30 seconds (alternately) with 2 minutes on ice between each burst. However, small samples for Western blot or SDS-PAGE were not subjected to lysozyme treatment.

2.4.4 Inclusion body preparation from bacteria.

The presence of inclusion bodies is easily visible under the light microscope (x100 oil phase) as bright bodies. In addition, the bacterial cells do not become translucent on sonication, like wild type cells, but remain opaque.

A simple density centrifugation procedure was used as a crude purification. After the required OD at 600nm was achieved the cells were harvested and lysed as described in Section 2.43. The lysate was spun at 3000g for 5 minutes to remove cell debris and unbroken cells. The supernatant was then spun at 18000g for 20 minutes to pellet the inclusion bodies and was then treated according to the requirements of subsequent procedures, such as purification schemes. The pellet contained a number of other components apart from the insoluble recombinant protein, one of which was DNA. The presence of DNA could be problematic in subsequent purification procedures due to its ability to readily trap protein and precipitate. This problem was overcome by the addition of 1-2mg/ml, final concentration, of DNAase1 (Sigma) at the same time as lysozyme.

2.4.5 Preparation of microsomes from rodent tissues.

Microsomal fractions were prepared from rodent tissues according to Meehan *et al* (1988). After homogenising the tissues on ice in phosphate/KCl buffer (buffer described in Section 2.42) the homogenate was spun at 11000g to remove cell debris, nuclei and mitochondria. The resulting supernatant then entered the density centrifugation process as described for subcellular fractionation of yeast (Section 2.42). These samples were generously donated by Dr.C.J.Henderson and Mr.T.K. Bammler.

2.5. Protein purification techniques.

The majority of these procedures were designed to recover active recombinant microsomal GST from inclusion bodies, however there are no defined protocols for achieving this and the procedures are example specific. The protocols used here are based on the purification of the native enzyme from rat liver microsomes (Morgenstern *et al* 1982a).

2.5.1 Solubilisation of inclusion bodies.

A powerful chaotrope was needed to solubilise the recombinant protein trapped in inclusion bodies; ie 8M urea and 10M guanidinium hydrochloride (Gn-HCl). These compounds were made up in the standard buffer (10mM potassium phosphate pH 7.0, 1% Triton X-100, 0.1mM EDTA, 20% glycerol and 1mM GSH). The 18000g pellet was stirred overnight at 4^0C and then centrifuged at 10000g for 30 minutes to remove insoluble material.

2.5.2 Gel filtration on Sephadex G-50.

A 2.5 cm by 36 cm column was packed with Sephadex G-50 (Pharmacia) and equilibrated with 6M Gn-HCl in standard buffer. Solubilised inclusion bodies were applied to the column, after the concentration of Gn-HCl was diluted to 6M with standard buffer, and run at 20ml/hour. The

presence of the recombinant protein was assessed by SDS-PAGE and Western blot analysis.

2.5.3 Hydroxyapatite chromatography.

The hydroxyapatite (Biorad HT) was mixed 1:1 with Sephadex G-25 (Pharmacia), this was to prevent the column from clogging due to heterogeneous suspension. The Gn-HCl was removed from the sample by dilution with standard buffer until 0.6M, then final traces were removed by dialysis. A 1.5x30cm column was equilibrated with standard buffer and the sample applied at a flow rate of 20ml/hr. The samples were eluted using a linear gradient of 10-250mM sodium phosphate in standard buffer. The presence of the recombinant protein was assessed by SDS-PAGE and Western blot analysis.

2.5.4 Ion-exchange chromatography on carboxymethyl Sepharose 6LB.

Cation-exchange chromatography was performed according to Morgenstern *et al* (1982a). The Gn-HCl was removed from the sample by dilution with standard buffer until 0.6M, then final traces were removed by dialysis. The sample was then applied to a 1.5 cm by 12 cm carboxymethyl (CM) Sepharose 6LB (Pharmacia) column, equilibrated with standard buffer at a flow rate of 20ml/hr. The protein was eluted in a single step by the application of 200mM KCl in standard buffer. The presence of the recombinant protein was assessed by SDS-PAGE and Western blot analysis.

2.5.5 Purification of *Schistosoma japonicum* glutathione S-transferase fusion proteins.

The fusion proteins were purified according to Smith and Johnson (1988) using a 1.5x4cm GSH affinity column:- Glutathione-agarose (sulphur linkage, Sigma). Six litres of LB were inoculated at a 1:20 dilution with a stationary overnight culture of fusion protein expressing bacteria, after

1 hour the culture was induced with 0.1mM IPTG. After 5-6 hours the cells were harvested and lysed, (Section 2.43), into PBS (Phosphate Buffered saline pH 7.3 Oxoid, Basingstoke, Hants) containing 0.1% Triton-X100, to reduce bacterial contaminants binding to the column. The lysate was spun at 10000g and the supernatant applied at a flow rate of 40ml/hr. to the column, which had been previously equilibrated with the PBS/0.1% TritonX-100 buffer. The 10000g pellet was gently agitated in a PBS/1% TritonX-100 buffer overnight in order to remove the fusion protein associated with this fraction. The mixture was then spun at 10000g and the concentration of Triton X-100 reduced to 0.1% with PBS and then applied to the column. After washing the column in buffer the protein was eluted with 5mM GSH in 50mM Tris-HCl pH 8.0. The purification procedure was followed using a UV detector at absorbance 280nm and the samples were assessed for CDNB activity to detect the presence of the fusion proteins.

2.5.6 Purification of protein A fusion proteins.

The protein A fusion proteins were purified in batch form, with a starting culture of 0.5-1 litre, using IgG-Sepharose 6 Fast Flow (Phamacia). The buffer used in this procedure was TST (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.05% Tween 20, 1mM GSH for the reasons discussed), as this was the same buffer in which the cells were lysed (Section 2.43). After sonication the lysate was spun at 10000g to remove debris, and then the supernatant was mixed overnight on a rotating wheel with 1ml of IgG-Sepharose, which had been previously equilibrated with TST. The IgG-Sepharose was pelleted by centrifugation and washed 8 times in TST, before addition of 4ml of elution buffer (0.5M acetic acid adjusted to pH 3.4 with ammonium acetate). Elution was carried out on a rotating wheel at 4^0C for 20 minutes, after which a second elution was performed for a further 10 minutes. The two eluates were pooled and dialysed against distilled water before lyophilisation. After freeze-drying, the protein was resuspended in a suitable buffer for thrombin cleavage or measuring GST activity and 0.1% TritonX-100/1mM GSH was included . The presence of the recombinant protein was assessed by SDS-PAGE and Western blot analysis.

2.5.7 Purification of phosphofructokinase A fusion proteins.

The phosphofructokinase (PFK) fusion proteins were purified in a batch form which was an adaptation of the column based procedure used to purify recombinantly expressed PFK as described by Lau *et al* (1987). The affinity matrix used was Cibracon blue (reactive blue, Sigma) and the buffer was a low salt buffer (50mM Tris-HCl pH 7.5, 0.1mM EDTA and 1mM GSH, used as antioxidant and thought to stabilise GSTs, and hence its replacement of β-mercaptoethanol used in the original method).

Cells were prepared as described in Section 2.43 and lysed in the low salt buffer, and after a 10000g clearing spin the supernatant was incubated overnight with reactive blue sepharose(Sigma), which had been pre-equilibrated with low salt buffer. The reactive blue was collected by centrifugation and washed with four volumes of high salt buffer (low salt buffer plus 1.5M NaCl) and the protein eluted with 2mM ATP/ 10mM $MgCl_2$. 0.1% Triton X-100 was added in one sample when eluting, while the other was detergent free, to provide a favourable environment for the microsomal GST after proteolytic cleavage. The presence of the recombinant protein was assessed by SDS-PAGE and Western blot analysis.

2.5.8 Proteolytic cleavage of the fusion proteins with thrombin.

All the fusion proteins were designed such that the microsomal GST could be released by cleavage at a unique thrombin site engineered 5' of the N terminus of the microsomal GST (all the fusion proteins were constructed with the microsomal GST C terminal of the carrier protein). In the case of the *Schistosoma Japonicum* GST fusion the thrombin site was present upstream of the multiple cloning site in the vector, pGEX-2T, However, with the other two constructs, the DNA sequence encoding the thrombin site was incorporated into the PCR oligo used to generate the microsomal GST cDNA. The fragment generated could then be directionally cloning into the protein A fusion protein vector, pAX, and subsequent construction of the microsomal GST fusion with PFK (details are discussed in Section 5.40).

The thrombin digest was carried out essentially as described by

Smith and Johnson (1988), with modifications. The fusion proteins were transferred in cleavage buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 2.5mM $CaCl_2$, 1mM GSH and 0.1% TritonX-100 to stabilise the microsomal GST) either by adjusting the existing buffer or by dialysing against cleavage buffer. For cleavage on the GSH-Agarose, however the 1mM GSH was omitted from the above buffer. Thrombin (from human plasma, activity 4000 NIH units/mg protein, Sigma) was added at a enzyme-to-substrate ratio of 1:500 and incubated for 1 hour at 37^{0}C. In order to carry out the thrombin digest on the affinity matrix the *Schistosoma Japonicum* fusion proteins were first reabsorbed onto GSH-Agarose in an eppendorf and then washed in PBS/TritonX-100 buffer. When no more CDNB activity could be measured in the washes, the buffer was changed to cleavage buffer.

2.5.9 Ion exchange using FPLC.

The MonoQ anion exchange column was used in a Pharmacia FPLC system which comprised a model GP-250 gradient programmer, two model P-500 pumps and a UV-1 UV monitor. The column was equilibrated with 20mM ethanolamine-HCl pH 9.0, 1mM GSH and run at a flow rate of 1ml/min. A 0.5 ml sample was injected and after 10 minutes buffer B (20mM ethanolamine-HCl pH 9.0, 1mM GSH,0.5M NaCl) was introduced over 40 minutes until the percentage of B was 40%, then the concentration was raised to 100% B over the remaining 10 minutes of the run. Protein peaks were detected using UV and 1ml samples were collected in a Pharmacia fraction collector and assessed for accurate protein concentration and CDNB activity.

2.6. Biochemical techniques.

2.6.1 Protein estimation.

The method of Lowry (1951) was used to measure the protein concentration in samples. A range of concentrations (0-200μg/ml) from bovine serum albumin (BSA) was used to generate a standard curve each time the assay was performed. However, for samples containing TritonX-

100 an appropriate concentration of detergent was included in the standards, as this detergent interferes with the Lowry assay. Samples were diluted 1:200 or 1:40 in order to fall within the concentration range of the standard curve. The plot of protein concentration against OD 600nm was approximately linear over the range 0-200μg/ml. A Shimadzu UV 160 spectrophometer was used to measure the optical densities, and each standard was measured in duplicate, while the samples were measured in triplicate.

2.6.2 Determination of glutathione S-transferase activity.

The method was essentially that of Habig *et al* (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as a model substrate for GST. Conjugation of this substrate was measured spectrophotometrically at 340nm. The sample was added to a cuvette containing 1mM CDNB in 100mM phosphate buffer pH 6.5 and 5mM GSH. The concentration of GSH was raised from 2mM, used for determining cytosolic GST activity, because of the higher Km value of the microsomal GST for GSH. The non-enzymic conjugation rate of GSH with CDNB was determined before the sample was added and subtracted, if significant, from the final result. Measurements were made using a Shimatzu MPS 2000 spectrophotometer and the change in absorbency was calculated per minute per mg of protein using the extinction coefficient $9.6x10^{-3}$.

The N-ethylmaleimide (NEM) activation of the microsomal GST was carried out by incubating 50μl of yeast microsomes (20-30 mg/ml) with 2mM NEM (final concentration) at room temperature for 2 minutes. The reaction was then terminated by adding an equivalent concentration of GSH (to the NEM). The sample was then assayed for CDNB activity.

2.6.3 Determination of P450 reductase activity using the cytochrome c.

The assay measured the NADPH reduction of cytochrome c by NADPH cytochrome P450 reductase. Cytochrome c (Sigma) (970μl) was

dissolved (1.5mg/ml) in 300mM potassium phosphate buffer pH 7.4, 20 μl microsomes were added to a 1ml cuvette and a base line obtained. NADPH was added at a final concentration of 160mg/ml and the change in absorbency measured at 550nm. The activity of the P450 reductase was calculated as change in absorbency at 550nm per minute per mg of protein using the extinction coefficient 0.0187. Measurements were made using a Shimatzu MPS 2000 spectrophotometer.

2.6.4 Measurement of protein concentration and GST activity in the fractions collected from the FPLC.

Both the protein concentration and CDNB assay were performed using the Cobras Fara centrifugal analyser (Roche Diagnostica, Welwyn Garden city, UK.) essentially as described by Hayes and Clarke (1982). For the measurement of CDNB activity, up to 29 samples were simultaneously pre-incubated with GSH, and the reaction was started by adding CDNB followed by automatic mixing by centrifugation. The final substrate concentrations were 1mM CDNB and 5mM GSH in a reaction volume of 200μl. The reactions were monitored by an initial measurement of absorbency at 340nm 10 seconds after mixing followed by 7 absorbency measurements at 5 second intervals. Linear regression analysis was preformed using the manufactures kinetic rate programme for the GST assay. A preprogrammed conversion factor was used and data from the CDNB assay was presented as a change in absorbency ΔA/minute/ml. the extinction coefficient (38) allowed the conversion of these units to mmol/minute/mg.

The Cobras Fara also determined the protein concentration by employing the Bradford assay (Bradford 1976).

2.6.5 Cytotoxicity testing of compounds in yeast.

The cytotoxicity assay was carried out as described by Black *et al* (1990). Cells were harvested in late Log. towards stationary phase when expression of the recombinant protein was maximal. Great care was taken to

insure that the control and expressing cultures were at similar points in the growth phase, so to minimise differences in sensitivity to the drug due different ages of the cultures. The exact cell density was determined using a heamocytometer and an aliquots of $4x10^7$ cells were taken and harvested by a 3000rpm spin in a Beckman D6 centrifuge. The cells were resuspended in 1.96ml of PBS. The test chemical was dissolved in a appropriate vehicle at 50 times the required final concentration,40μl was then added drop wise to the cell suspension. Control samples contained 40μl of vehicle. The cells were then incubated at the desired temperature, with shaking, for two hours. After incubation the drug was removed and the cultures were serially diluted into 0.85M NaCl to a final concentration of 200 cells per 100μl (as calculated from the starting culture). Aliquots of 100μl were plated in triplicate onto YPD agar plates and incubated for 3 days at 28°C. A colony counter was used to determine the number of cells on each plate and the results expressed as a percentage survival of the control.

2.7. Sodium dodecyl sulphate polyacrylamide gel techniques.

2.7.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was carried out according to the method of Laemmli (1970). The stacking portion of the gel consisted of a 4.5% w/v acrylamide gel, but for the separating gel, the percentage of acrylamide varied according to the molecular weights of the samples being resolved. For lower molecular weights (range 10-40KDa) a 12% gel was used, where as for higher molecular weights (>50KDa) a 9% gel was used. Where two proteins with similar molecular weights, in the region of 17KDa, were to be resolved gradient gels of 10-15% were poured.

The samples were prepared at 2x the required concentration and diluted 1:1 with 2x boiling mix (50mM Tris-HCl pH 6.8, 2% w/v SDS, 5% w/v mercaptoethanol, 10% w/v glycerol, 0.05% w/v bromophenol blue) and boiled for 5 minutes.

The samples were run through the stacking gel at 50mA and 25mA

through the separating gel. The apparatus was water cooled. After electrophoresis the gels were removed and either stained in order to view the proteins or subjected to Western blot analysis.

2.7.2 Coomassie blue staining of SDS-PAGE.

The gels were stained and fixed in 0.25%w/v Coomassie blue R, 10% acetic acid, 40% methanol for 30 minutes to 1 hour, after which they were destained in 10% methanol, 7% acetic acid.

2.7.3 Silver staining of SDS-PAGE.

This method is a considerably more sensitive means of protein detection than coomassie blue and was employed when protein concentrations were low. In addition, TCA precipitation (2 times the volume of 40% trichloroacetic acid, 20 minutes on ice and 6000rpm for 20 minutes in a bench top centrifuge) also helped to concentrate samples and enhanced sensitivity.

Gels were fixed for 1 hour in 50% methanol, 12% acetic acid, and 0.05% formaldehyde, after which they were washed 3 times in 50% ethanol for 10 minutes. Pretreatment consisted of 1 minute in 0.02% sodium thiosulfate ($Na_2SO_2O_3.5H_2O$) followed by a 3 times 20 second wash in distilled water. The gels were then impregnated with 0.2% silver nitrate ($AgNO_3$) and 0.075% formaldehyde for 20 minutes and rinsed again in distilled water. The development of the gel was carried out by the addition of 6% sodium carbonate (Na_2CO_3), 0.05% formaldehyde, 0.004% sodium thiosulfate ($Na_2SO_2O_3.5H_2O$). When the protein bands had reached the desired intensity the gels were rinsed in distilled water and the reaction stopped by adding 50% methanol, 12% acetic acid for 10 minutes. The gels were stored in 30% methanol awaiting photography.

2.7.4 Western blot analysis.

Western blot analysis was performed by a modification of the method according to Towbin *et al* (1979). Following SDS-PAGE the gel was

transferred, according to the manufactures instructions, onto a 0.45mm nitrocellulose filter (Schleiser-Schuell) in a Biorad protean II transblot cell containing transblot buffer (20mM disodium ortho-phosphate, 20% methanol) for 12 hours at 250 mA. After transfer the filter was washed in TBST (50mM Tris-HCl pH7.9, 0.15 NaCl, 0.05% Tween 20) for 2 times 10 minutes and then blocked in 3% low fat powdered milk (Marvel-Cadbury's) for 45 minutes. After blocking the filters were washed in TBST for 2 times 10 minutes, and then the primary antisera was applied (generally at a 1:500 dilution in TBST) for 1 hour. The nitocellulose filters were then washed for 2 times 10 minutes, followed by 2 times 5 minutes. The second antisera, HRP-conjugated with anti-rabbit IgG (as all first antisera were raised in rabbits), was applied for 1 hour then washed as before. To visualise the immunoreactive proteins, the filter was exposed to the HRP substrate 4chloro-1-naphthol in 40ml of methanol, 200ml of TBS (TBST minus tween 20) and 80μl of 30% hydrogen peroxide. After 10 minutes the reaction was terminated by washing in distilled water.

Radiolabelling of blots was carried out by the addition of 0.19 MBq of ^{125}I-conjugated protein A (Amersham International) to the filter in 50ml of TBST. After an hour incubation the iodine was removed and the filter repeatedly washed in TBST until the γ counts remained constant. The filter was air dried and exposed to autoradiographic film.

2.7.5 Antisera used in the detection of the microsomal glutathione S-transferase.

Three antibodies were available for detecting the presence of the recombinant protein in yeast and they were all polyclonal antisera. Initially two antisera were used, they were raised against purified rat and human microsomal protein by Dr. Lesley McLellan (McLellan 1989). The anti-rat microsomal GST antibody cross reacted with a band in the wild type yeast at a similar molecular weight as the expressed microsomal protein, 17.3 KDa. This is clearly illustrated in Figures 4.5a and Appendix B. Resolving these bands were problematic, and the best results were achieved by running 10-15% gradient SDS-PAGE. However longer exposure on autoradiographs

caused these bands to merge, as seen in Figure 4.14 and 4.15. This cross-reacting band did not appear when the anti-human microsomal GST antibody was used.

In Section 6.3.6 a second polyclonal anti-rat microsomal GST antibody was prepared. In order to obtain antigen for the raising of this antibody, the rat microsomal GST was expressed in *E.Coli* (anti-rec microsomal GST antibody). This antibody failed to cross react with any native yeast band at the previously observed molecular weight. The original anti-rat microsomal GST antibody was immunopurified by Dr. Derek Jamieson using recombinant expressed microsomal GST fusion proteins, (described in Section 6.3.7). The immunopurified anti-rat microsomal GST antibody no longer cross-reacted with any yeast protein, thus illustrating the non-specific nature of this cross-reaction.

Chapter 3. Investigation of the endogenous regulation of the rat and mouse microsomal glutathione S-transferase.

3.1. Introduction.

The major forms of regulation that characterises drug metabolising enzymes is their induction by xenobiotics. However, the microsomal GST does not appear to be greatly affected by this form of regulation (Morgenstern *et al* 1982a).

Other modes of regulation are also involved in modulating the expression of drug metabolising enzymes. For example expression differences between species; distribution amongst tissues and sexual dimorphism have all been reported (Zaphiropoulos *et al* 1989, and Skett 1987).

3.1.1.The endocrine system — a brief overview.

The endocrine and nervous system control the adjustments the body makes in response to environmental changes. The endocrine system mediates its control via hormones circulating in the blood. Some hormones, for example growth hormone act on several tissues rather than one target organ, implying the receptors for these hormones are wide spread. Other hormones have a single target tissue, for example thyrotrophin, adrenocorticotrophin (ACTH) and gonadotrophin, which are secreted by the anterior pituitary and act on the thyroid gland, adrenal cortex and gonads, respectively. Table 3.1 summaries the major hormones produced by the pituitary and their actions.

Regulation of hormonal release from the anterior pituitary gland is complex. Pituitary secretion is modulated, in some cases, by hormones produced by target organs in a negative or positive feedback. In addition the hypothalamus exerts hormonal control on the pituitary gland.

Growth hormone is produced in the pituitary somatotrophs. Release of this hormone is under hypothalamic control via growth hormone releasing hormone (GHRH) and somatostatin acting in a positive and negative manner respectively. Growth hormone has a wide

Table 3.1 Hormones produced by the pituitary gland and a summary of their actions.

Name and source	Action.
Anterior lobe.	
Thyroid-stimulating hormone (TSH, thyrotropin)	Stimulates thyroid secretion and growth.
Adrenocorticotropic hormone (ACTH< corticotropin)	Stimulates adrenocortical secretion and growth.
Growth hormone (GH, somatotropin, STH)	Accelerates body growth.
Follicle-stimulating hormone (FSH)	Stimulates ovarian follicle growth in the female and spermatogenesis in the male.
Luteinizing hormone (LH> interstitial cell stimulating hormone, ICSH)	Stimulates ovulation and leuteinization of ovarian follicles in female and testosterone secretion in males.
Prolactin (luteotropic hormone, LTH, luteotropin lactgenic hormone, mammotropin).	Stimulates secretion of milk and maternal behaviour.
β-Lipotrophin	Involved in stress response.
Intermediate lobe.	
α- and β- Melanocyte-stimulating hormones (α- and β- MSH; referred to collectively as melanotropin or intermedin)	Expands melanophores.
Posterior lobe.	
Vasopressin (antidiuretic hormone, ADH)	Promotes water rejection.
Oxytocin	Causes milk secretion.

range of metabolic effects in tissues, manifested in the stimulation of synthesis of proteins and nucleic acids. One of the major proteins is somatomedin C/insulin like growth factor (SMC/IGF1). This is produced in many tissues but 50% is synthesised in the liver (D'Ercole 1984). SMC/IGF1 is thought to mediate growth hormone action, initiating DNA synthesis and cell proliferation. In cell culture it has been shown to act synergistically with platelet derived growth factor (PDGF) in promoting growth. (Stiles *et al* 1979).

The hypothalamus also stimulates production of thyrotrophin by the secretion of thyrotrophin releasing hormone (TRH). Thyrotrophin binds to a specific receptor on the follicular cell surface of the thyroid and activates release or the thyroid hormones, thyroxine (T4) and triiodothyronine (T3).

Adrenocorticotropic hormone (ACTH) is a 39 amino acid polypeptide, whose action on the adrenal cortex stimulates the synthesis of steroids. Interaction of ACTH with its receptor on the adrenocortical plasma membrane brings about activation of a cAMP dependent protein kinase (Schimmer 1980; Bost and Black 1986). Short-term action of ACTH stimulates the conversion of cholesterol to glucocorticoid, mineralocorticoid and androgen precursor steroids. (Hornsby 1988). In the longer term ACTH effects affects the synthesis of enzymes involved in steroidogenesis.

3.1.2.The regulation of drug metabolising enzymes by the endocrine system.

The endocrine control of xenobiotic metabolism is mainly directed at the liver and kidney. This is not surprising as highest levels of drug metabolising enzymes are located in the liver (review see Skett 1987)

Developmental regulation of some proteins is under hormonal control. This can be seen by the examination of P450 gene expression during development. In rats CYP2EI, CYP2DI and CYP2D2 mRNA levels are elevated immediately after birth. (Gonzalez *et al* 1987. Song *et al* 1986). In the case of CYP2E1 the transcriptional activation correlates with changes in the methylation state of the gene (Umeno *et al* 1988).

The inducibility of these genes is also developmentally regulated. The aromatic hydrocarbon 2,3,7,8 tetrachloro-benzo-p-dioxin (TCDD) induces the expression of mouse Cyp 1a1 gene at 10 days gestation, whereas Cyp1a 2 is not induced until 1 week after birth. (Ikeda *et al* 1983). In the rat, CYP1A1 and 1A2 are not inducible until one week after birth (Giachelli and Omieciniski 1987). This exemplifies a species specific difference in developmental regulation.

Some xenobiotics show a sex-specific metabolism which may be explained by a sexual dimorphism in the expression of the enzymes involved in their metabolism. The pharmacological and toxicological consequences readily manifest themselves (Zaphiropoulos *et al* 1989). Again the question of a possible endogenous role of the drug metabolising enzymes is raised. A number of the P450s are involved in steroid biogenesis, a role which has also been proposed for the GST's (Ketterer *et al* 1990). A sex differences in these enzymes may well be involved in maintaining the appropriate endocrine balance.

It has been proposed that the control of sexual dimorphism of some drug metabolising enzymes, such as P450, occurs via growth hormone (Zaphiropoulos *et al* 1989). Secretion of growth hormone has a sex-specific pattern. In the male rat it is intermittent, while in the female it is continuous. By this means growth hormone directly, or via the somatomedins, leads to the induction or suppression of various proteins in a sex-specific manner. This sex-specific secretion of growth hormone is thought to be brought about by a brief surge of testosterone soon after birth. The evidence for this so called "neonatal imprinting " comes from castration and hormone replacement studies. Only neonatal castration can cause a complete female pattern of expression, while only testosterone administered directly after castration can replace the male phenotype. However, other observations show that testosterone treatment is not always capable of restoring the wild type levels of protein expression. This questions the growth hormone hypothesis and other factors, such as sex steroids, have been implicated. Steroids can also interact with hypothalamic factors, and it has been shown-that steroids can directly control hepatic protein expression. Oestrogen receptors are found in both male and female liver. (Wrange *et al* 1980). Androgens also

have been reported to directly stimulate α2 microglobulin synthesis in perfused rat liver (Murty *et al* 1987).

Some drug metabolising enzymes are independent of the growth hormone secretion pattern. For example the level of 6β hydroxylation of steroids by CYP 3A1 and CYP 3A2 is reduced by both continuous (female) and intermittent (male) administration of growth hormone (Yamazoe *et al* 1986). The induction of CYP 2B1 and CYP 2B2 by phenobarbital is increased by hypophysectomy and repressed by growth hormone (Yamazoe *et al* 1987) .

3.1.3 The endogenous regulation of the cytosolic glutathione S-transferases.

The cytosolic GSTs show marked species differences in expression and sexual dimorphism, the underlying mechanisms of which have received much attention.

The YfYf (7-7) class GST is the major form of GST in the male mouse liver (Hatayama *et al* 1986; McLellan and Hayes 1987). The level of expression of this form of GST is gradually increased during development in the male, while remains unchanged in the female, resulting in a tenfold higher level of expression in the adult male. Neonatal castration of the male mouse leads to a feminised pattern of expression, while administration of testosterone to females raises the Yf protein expression to male levels (Hatayama *et al* 1986). These results indicate that the expression of the π class GST is developmentally regulated by testosterone in the male mouse. Further work demonstrated that testosterone exerts its effect on YfYf expression indirectly, via the pituitary gland (Dolan 1990). Studies with the growth hormone deficient strain of mice, the little mice, implicated growth hormone in mediating the effect of the pituitary gland.

The pattern of pattern of expression of the individual GST isoenzymes was found to be different in the rat. The μ class subunit, Yb_1, was expressed at higher levels in the male rat liver than female, as judged by the 2-3 fold higher DCNB activity in the male (Hales and

Niems 1979). This observation was confirmed by Igarashi *et al* 1985 and in addition, the female liver was found to contain higher levels of the α class GSTs. The sexual dimorphism with regard to the μ class expression could be eliminated by hypophysectomy (Lamartiniere 1981). The administration of both somatotropin and monosodium-L-glutamate, the latter of which is known to prevent growth hormone release by destroying the arcuate nucleus in the hypothalamus, decreased the male level of Yb expression (Lamartiniere 1981). Again, as in the mouse, the hypothalamic-hypophyseal-gonadal axis appears to be exerting an effect on GST expression in the liver.

Ligandin, comprising of the α class GST subunits Ya and Yc (subunits 1 and 2), shows a tissue specific pattern of developmental regulation (Eidne *et al* 1986). In the steriod-synthesising tissues; testis, ovary and adrenal gland; were relatively high after birth, decreased by day 9 and increased rapidly during puberty to reach adult levels. These changes appeared to be parallelled by changes in the circulating levels of testosterone and progesterone. In contrast, the levels of this protein the non-sterogenically active tissues, such as liver and kidney, was low at birth and increased gradually to reach adult levels. In addition hepatic levels could be induced with phenobarbital at all stages of development, whereas no change in expression was noted in endocrine tissues following such treatment.

The adrenal cortical cells being responsible for the synthesis of steroid hormones, while the medulla for the secretion of adrenaline and noradrenaline. The GSTs have been implicated in both the binding and storage of steroids. (Homma *et al* 1986). Also, they are known to have conjugating activity to endogenous epoxides, such as cholesterol epoxide, and other lipid products. (Meyer and Ketterer 1982; Ålin *et al* 1985).

The regulation of the adrenal GSTs would appear to be dissimilar from the liver population. Unlike the liver, the enzymes found in the adrenal are not inducible by 3 methylcholanlthrene and phenobarbital. (Bengtsson *et al* 1983). The pituitary gland appears to have a role in basal GST expression. Hypophysectomy causes an increase in a number of isoenzymes in the rat, namely subunits 2,3,4,7, and 8. Each

isoenzyme has its own distribution pattern within the adrenal infrastructure, which is altered after hypophysectomy (Mankowitz *et al* 1991a). The significance of the distribution of these enzymes is worth considering in light of their individual role. GST subunit 4 was studied in further detail, the observed increase following hypophysectomy being subsequently reversed by administration of adrenocorticotrophin. (Mankowitz *et al* 1991b) .

Adrenocorticotrophin, a pituitary hormone regulates the level of a number of enzymes in the adrenal gland, for instance members of the P450 family involved in steroid and xenobiotic metabolism. (Guenthner *et al* 1979). Further investigations have shown these genes posses a cAMP-responsive regulatory elements (Lund *et al* 1990 Rice *et al* 1989 and Kagawa and Waterman 1990). Some indirect evidence was presented in the study on GST subunit 4, suggesting that cAMP could also be involved in mediating the regulation of GSTs by adrenocorticotrophin. (Mankowitz *et al* 1991b).

3.1.4.Exogenous regulation of the cytosolic glutathione S-transferases.

The majority of the work in this area has focussed on the liver. As mentioned previously the effects of xenobiotics can be tissue specific, and it is possible that several regulatory mechanisms may be operating.

A number of common P450 inducers such as phenobarbital, dexamethasone and polycyclic aromatic hydrocarbons (PAH) also induce the GST's. Since many P450 metabolites are substrates for GSTs, this would make sense in evolutionary terms. In chapter 1, a similar idea was applied to the evidence that P450 metabolites were responsible for the direct activation of the microsomal GST. In both the P450 and GST systems, the PAH induction mechanism has been characterised by RNA blot hybridisation and nuclear run-on experiments. The results demonstrate that the Ya and Yb subunits are transcriptionally activated by 3 melthylcholanthrene (a PAH) and phenobarbital (Ding *et al* 1985).

The 5' flanking region of the Ya gene has been analysed.

(Telakowski-Hopkins *et al* 1988) and found to contain a xenobiotic responsive element (XRE). This sequence is also found in the P450 1A1 5' region and the core sequence is :-

5'-T- GCGTG-3'

3'-A-CGCAC-5'

was found in multiple copies in the P4501A1 gene. The Ah receptor is responsible for mediating the transcriptional response to PAH via this XRE (Denison 1989). The details of this pathway and elements involved have in part been elucidated. (Hankinson *et al* 1992). In the case of the Ya gene the XRE is located in a single copy in the region -908 to -899 bp and is only partly responsible for response to PAH (Rushmore *et al* 1990). A second element at -722 to -682 bp exists which is responsive to PAH and is also involved in basal activity of the gene. Chloramphenicol acetyl transferase (CAT) studies using cell lines deficient for Ah receptors demonstrate that this region does not require an Ah receptor and is distinct from XRE found in P450 1A1 (Rushmore and Pickett 1990). This sequence 5' ggTGAaaaGC-3' is responsive to PAH and the corresponding P450 metabolites - phenolic antioxidants (Rushmore and Pickett 1990). This unique XRE ,termed the Antioxidant Responsive Element (ARE), was also responsive to hydrogen peroxide, which suggests a mechanism of gene activation by reactive oxygen species (Rushmore *et al* 1991). Recently the trans-acting factors for the ARE have been identified as a protein heterodimer with subunit molecular weights of 28KDa and 45KDa (Nguyen and Pickett 1992).

The π class GSTs, rat 7-7 and human π, have been extensively studied due to their over-expression in some examples of malignant transformation; (Satoh *et al* 1985 and Li *et al* 1988) and their involvement in drug resistance (Batist *et al* 1986) In the rat, true enhancer elements exist at -2.5 and -2.2 kilobases (Kb), were designated GPE11 (Okuda *et al* 1989). The GPE1 site contains the sequence for 12-O-tetradecanoyl phorbol 13-acetate (TPA) responsive element TRE (Sakai *et al* 1988 Tahir *et al* 1989). Not only can the rat 7-7 gene be induced by TPA, but also by c-Ha-ras and N-ras oncogenes (Power *et al* 1987, Li 1988; Li and Liberman 1989). Fos and Jun oncogenes are also known to act via TRE/API sites. Although it has not been proven that the rat 7-7 gene is a

target for the ras oncogene family, it is known that these oncogenes can induce phenotypic alterations resembling those seen in premalignant lesions (Bishop 1987). GPEII site contains two enhancer elements similar to those found in SV40 and polymovirus. However when this human gene was studied, the TRE was found to be present but not active (Dixon *et al* 1989).

Many other compounds can act to induce members of the GST gene family. The mechanisms are not as well understood as the examples cited here. Phenobarbital, another P450 inducer increases levels of Ya and Yb in the rat. The PAH responsive elements differ in the P450 and GST genes studied, however it will be interesting to see whether the GST genes the same regulatory sequences as the P450 genes in response to phenobarbital. Examples of other agents capable of inducing GST expression are *trans*-stilbene oxide, a GST substrate (Reference) and 2(3)-*tert*-butyl-4-thydoxyanisole (BHA) and 2(3)-*tert*-butyl-4-hydroxytoluene(BHT) (McLellan and Hayes 1990).

In comparing the regulation of the microsomal GST and cytosolic GST's discussion has focused on the former being a target for post-translational modification where as the cytosolic GST's are regulated at the transcriptional level. However, some post-translational modification of the cytosolic GSTs does occur. It has been demonstrated that rat isoenzymes are substrates for protein kinase C-dependent phosphorylation (Pyerin *et al* 1987) and for calmodulin-mediated methylation. (Siegel *et al* 1990). The physiological significance of these interaction has not been established, although the latter example may be sensitive to changes in calcium homeostasis within the cell as discussed in Chapter 1.4.

3.1.5.Summary

The overall understanding of the mechanisms that regulate P450 and GST gene expression are incomplete. The overview, presented here is meant to highlight some of the better characterised examples, with particular reference to the glutathione S-transferases.

Some xenobiotic metabolising enzymes show a sexual

dimorphism in their expression. The reason for the sex differences in protein expression is not understood, nor is it confined to the drug metabolising enzymes.

The induction of the phase I and II drug metabolising enzymes by xenobiotics, their substrates, is an efficient regulatory mechanism with an obvious function. The elegance of this form of regulation was illustrated by the induction of P450 1A1 and GST subunit Ya gene expression by PAH compounds. However the microsomal GST expression cannot be induced by xenobiotics, but the significance of abstaince from such an apparently effective regulatory mechanism is unclear.

3.2. Aims of the chapter

It is the aim of this Chapter to investigate the distribution of the microsomal GST protein expression in the whole animal. Much information about the function of a protein can be gathered from such studies. For example, the highest level of expression of many of the drug metabolising enzymes is in the liver, and this is thought to be because this organ is the major site of metabolism in the body.

In addition, the involvement of endogenous regulation of the microsomal GST, by the pituitary, will be investigated by using a range of rodent models. The hormonal regulation of other phase I and II enzymes has already been discussed in the previous section and will be used as a comparison. If the microsomal GST shares a common role with these proteins then a similarity in regularity mechanisms might be expected.

3.3. Strategies

3.3.1.Tissue distribution of the rat microsomal glutathione S-transferase.

Valuable information can be obtained as to the function of a particular protein by assessing the tissue distribution. Microsomes were prepared by differential centrifugation (Meehan *et al* 1988) from liver, testis, lung, large and small intestine, kidney, heart, muscle, spleen, and brain.

3.3.2. & 3.3.3. The development and pituitary gland regulation of the mouse and rat liver microsomal glutathione S-transferase.

The pituitary gland has been shown to be responsible for the regulation of a number of enzymes involved in drug metabolism and steroid synthesis (Skett 1987). In addition sexual dimorphism in enzyme expressions mediated by this organ. Two strategies were employed to assess the involvement of pituitary regulation in the expression of the microsomal GST.

Firstly, changes in protein expression during the development will establish if the pituitary gland is involved in regulating expression. Microsomes were prepared from male and female mice at two, four, six, eight, and ten weeks old (twelve of each sex of two and four weeks; six of each sex at six, eight and ten weeks).

Secondly, to assess the direct effect of pituitary control the organ was removed by intra-auricular hypophysectomy at the age of six weeks. Control animals were sham-operated to simulate the operation stress without removing the pituitary, this was carried out in mice and rats. To assess the success of the operation the weight of the animals was monitored over a two week period. Hypophysectomised animals failed to gain weight in contrast to the sham-operated mice, which developed normally.

At the age of nine weeks hypophysectomised and sham-operated

mice (six of each sex and treatment group) were given an intraperitoneal injection of phenobarbital (80mg/kg body weight per day for three consecutive days in phosphate buffered saline (PBS)) or dexamethasone (100mg/kg body weight per day for four consecutive days in corn oil). Control animals were injection vehicle only.

3.3.4.The role of growth hormone in the regulation of the mouse liver microsomal glutathione S-transferase.

The pituitary gland secretes a number of hormones with different target sites and effects (section 3.11), and growth hormone has been implicated in regulating the expression of a number of hepatic proteins.

Several mutant mouse strains deficient in a range of endocrine functions are now used quite extensively to investigate hormone related phenomena as reviewed by Charlton (1984). The mutant little mouse strain was derived from C57BL/6 (Beamer and Eicher 1976). This strain lacks a functional receptor for the growth hormone releasing hormone, so the synthesis of growth hormone is blocked. (Clark and Robinson 1983). The growth hormone levels in little mice are approximately 5% of normal levels. (Cheng *et al* 1983). The effect is inherited in an autosomal recessive fashion. Mice homozygous (lit/lit) for this recessive mutation are 60% in size of the wild type mice (+/+).

3.3.5. Acknowledgements and declaration.

I would like to express my grateful thanks to those mentioned below for the preparation of samples
Dr. Colin J. Henderson:- preparation of microsomes and RNA in Section 3.3.1, 3.3.2, 3.3.3 and 3.3.4.
Mr. Theodore K. Bammler:- preparation of microsomes in Section 3.3.2.
Dr. David J. Harrison:- performing immunohisto-chemistry in Section 3.4.1.

3.4. Results and discussion.

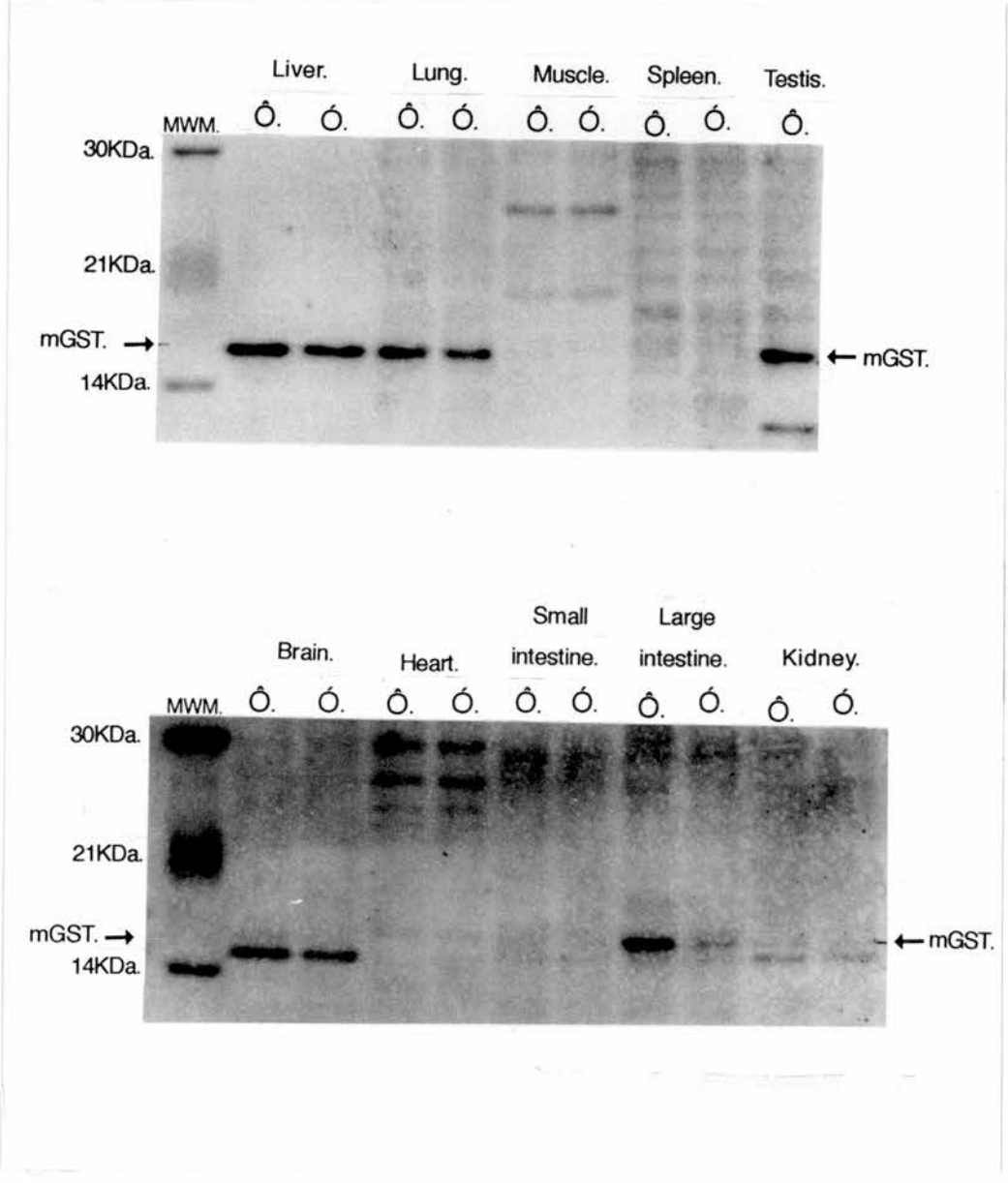
3.4.1.Tissue distribution of the rat microsomal glutathione S-transferase.

The tissue distribution of the microsomal GST was examined in adult male and female Wistar rats and the results of the Western blot analysis are shown in Figure 3.1. The highest level of expression is found in the liver and the level of expression in the male is slightly higher than the female. The results were quantified by densitomter-scanning of the Western blot, the data is shown in Table 3.2. The highest level of expression of the microsomal GST, found in an extrahepatic tissue, was in the lung, being approximately a tenth of the liver value (male=10.51% and female=8.96% of the liver value). The microsomal GST has been implicated in the protection of the cell against oxidative stress. The observation that the microsomal GST is highly expressed in this organ, relative to other extrahepatic tissues, would lend further support to this argument.

The large intestine exhibits a sexual dimorphism microsomal GST levels being 3-4 fold higher in the male, however all the tissues show slight expression differences between the sexes. In an immunohistochemistry study of the human intestinal epithelium only trace amount of microsomal GST were found (Hayes *et al* 1989) it would be interesting to determine the location of the microsomal GST in the intestine.

Figures 3.1 show the entire blot and demonstrates the presence of a number of bands in the 17 KDa region, as mentioned a polyclonal antisera was used. Affinity purification of the antiserum would eliminate non-specific cross-reactivity, however not enough antiserum was available for this purpose. In chapter 6 a recombinant protein was used to prepare a new antibody, then sufficient serum was available to affinity purify the antibody. However at the time of this work the antibody derived from the recombinant antibody was not available. An immuno pure antibody would have undoubtedly removed the problem of cross-reactivity. However, time did not permit the repetition of these

Figure 3.1. Tissue distribution of the rat liver microsomal glutathione S-transferase.



mGST: microsomal GST.

MWM: molecular weight markers.

Ó: female.

Ô: male.

3μg of protein of liver microsomes were loaded.

30μg of protein of microsomes from other tissues were loaded.

Table 3.2 Tissue distribution of the rat microsomal glutathione S-transferase.

Organ	Section 3.41 % protein male	Section 3.41 % protein female	Morgenstern *et al* (1984). %CDNB	%CDNB +NEM	ΔCDNB after NEM	%protein	DeJong . *et al* (1988). %mRNA	Ketterer *et al* (1990) Cytosolic GSTs %CDNB Rat	Human
Liver	100	100	100	100	5.50	100	100	100	100
Large intestine	8.69	2.44	47.8	5.34	6.62	12.6	ns	3.38	15.96
Adrenal gland	ns	ns	40.95	5.88	0.79	10.3	ns	5.90	94.12
Testis	9.50	-	102.38	18.42	1.00	8.1	20	4.25	81.50
Thymus	ns	ns	3.47	0.98	1.58	3.5	ns	ns	ns
Lung	10.51	8.96	12.22	2.50	1.16	2.6	8	4.10	28.57
Spleen	<0.1	<0.1	7.15	0.85	0.66	1.6	1	4.37	31.93
Kidney	0.17	0.33	6.78	1.21	0.98	<0.09	20	1.21	106.72
Brain	ns	ns	6.31	0.08	0.07	<0.09	8	9.32	76.47
Heart	<0.1	<0.1	5.81	0.05	0.51	nd	ns	3.67	38.65
Muscle	<0.1	<0.1	ns	ns	ns	ns	ns	1.41	86.55
Small intestine	<0.1	<0.1	ns	ns	ns	ns	ns	4.10	24.36

ns= not studied
nd=not determined
CDNB=CDNB activity measurements,
Protein=immunological determinant of protein present (Densitometric scanning).
All values expressed as a percentage of the liver value.

except Δ CDNB after NEM treatment = $\frac{\text{CDNB activity of NEM treated microsomes}}{\text{CDNB activity of untreated microsomes}}$

Morgenstern *et al* (1984) Biochem. Pharmacol. **33** 609-3614.
Dejong *et al* (1988) J.Biol.Chem. **263** 6430-8436
Ketterer *et al* (1990) Chapter 3 Soluble glutathione S-transferase isoenzymes. In: Glutathione conjugation mechanisms and biological significance. Academic press, New York.

experiments.

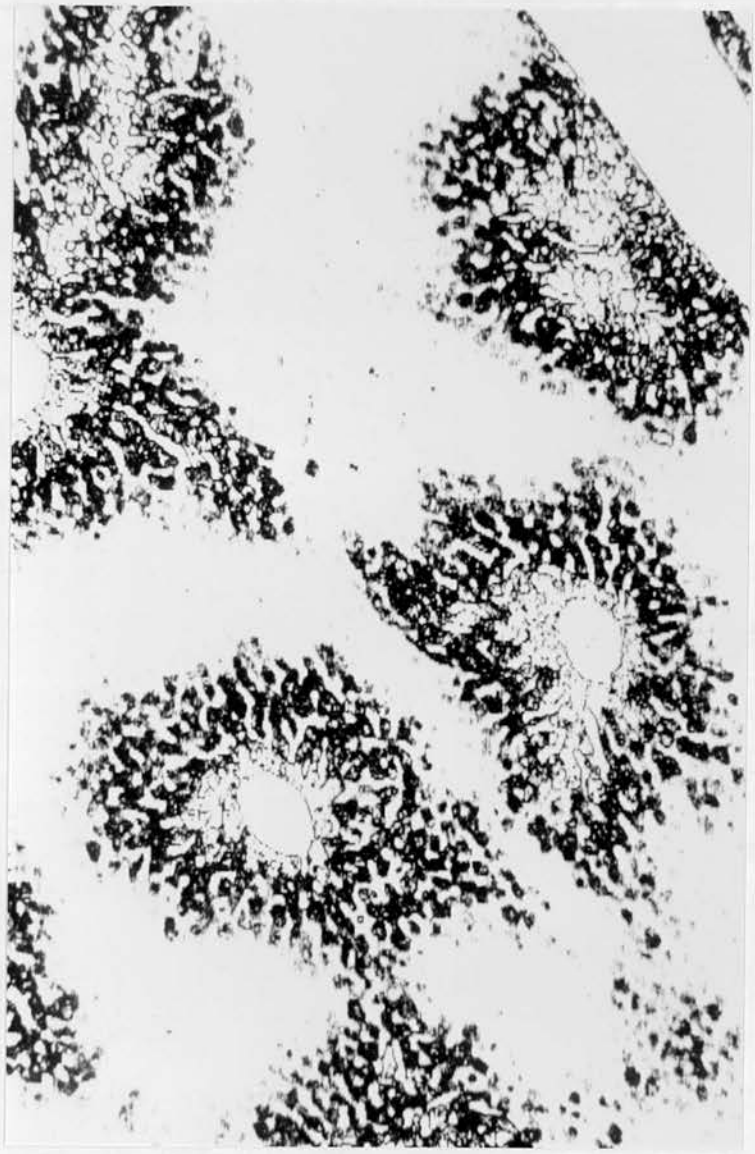
Immunohisto-chemistry of the rat liver was performed and is shown in Figure 3.2. The microsomal GST is located around the terminal hepatic vein in zone 3, this location is shared with the other drug metabolising enzymes such as the P450s and cytosolic GSTs. This observation again suggests the microsomal GST has a role in drug metabolism.

Discussion.

A similar study was carried out in by Morgenstern *et al* 1984 and the immunological data agree in broad terms with this study, despite the strain difference in the rats used (Table 3.2). Interestingly, the adrenal gland shows high extrahepatic expression of the microsomal GST. The GSTs have been implicated in steroid biogenesis (Ketterer *et al* 1990) and in the storage and binding of steroids (Homma *et al* 1986). Further support of these ideas come from the observation that steroid derivatives are inhibitors of the cytosolic GSTs (Tahir *et al* 1985) and the microsomal GST (Morgenstern and DePierre 1985). In contradiction, to the results of this study, the level of the microsomal GST in the lung is low (2.6% of the liver value) in the data presented by Morgenstern *et al* 1984. Sprague dawley rats were used and may explain the different observations, however this result detracts from the hypothesis that the microsomal GST is important in protection against oxidative stress.

Three criteria were used to assess microsomal GST content in tissues. Activity measurements using CDNB as a substrate before and after treatment with NEM were performed. Immunological analysis was also carried out, but showed discrepancies with the activity measurement. In this study no activity measurements were made as the tissue had been frozen for sometime. Freezing the tissue affects the activity and the response to NEM (Morgenstern *et al* 1980). The anomalies between activity and immunological data are best illustrated in the case of the testis. The activity observed in this organ is 102% of that in the liver, where as the amount of protein is only 8% of the liver value and no increase in activity was observed following NEM treatment.

Figure 3.2 Liver localisation of the rat liver microsomal glutathione S-transferase.



Localisation of the rat microsomal GST in the liver was demonstrated by immunohistochemistry. Sections were incubated with the polyclonal antibody binding (diluted 1:50 in TBS/5% swine serum) overnight at $4^{o}C$. Detection of antibody was performed using biotinylated swine anti-rabbit antiserum and an avidin-biotin-peroxidase system. Visualisation was by 3-3 diaminobenzidine. Sections were examined by light microscopy using a Zeiss Kontron and digital enhancement of the resulting image to more readily demonstrated the zonal pattern of the enzyme expression.

In fact in general no increase in activity following NEM treatment is observed in extrahepatic tissue (a slight increase is observed in the lung and thymus). The authors suggest that the protein in these tissues, namely adrenal, testis and intestine, may already be activated. This not wholly convincing since the adrenal and intestine show a significant decrease. Another possible explanation is contamination with cytosolic GST's which are known to be inhibited by NEM (Morgenstern *et al* 1982). No marker enzymes were used to determine the contamination of microsomes with other organelles. Morphological and physiological differences between tissues may not allow microsomes to be prepared in a uniform manner.

Another possibility is that there are other microsomal enzyme(s) with GST activity. Two separate reports have described the purification of GST activity from rat liver and brain (Dierickx *et al* 1986, Asano *et al* 1990). The activity in the microsomes was not removed by washing and could only be released by treatment with detergent Lubrol px or Triton X-100. The proteins were also purified by affinity chromatography on S-hexyl glutathione or GSH Sepharose 6B. The microsomal GST described by Morgenstern does not bind to affinity columns (Morgenstern and DePierre 1985) . The proteins purified from rat brain had molecular weights in the range 28-25 KDa similar to the cytosolic GST's (Mannervik and Danielson 1988).

In another study a Walker 256 mammary carcinoma cells made resistant to chlorambucil were found to have a 29KDa microsome - associated GST. This 29 KDa species was not found in the parent cell line, but cross-reacted with sigma antisera to rat liver cytosolic GSTs (Clapper and Tew 1989). If this species was not contamination of the microsomes with cytosolic GST's, then another factor must have caused this relocation. A possibility is post-translational modification, causing an increase in affinity for a membrane environment.

The tissue distribution of the microsomal GST was evaluated by analysis of the total amount of messenger RNA (mRNA) (DeJong *et al* 1988). Northern blot analysis does not account for translational differences between tissues,, and so it is not an absolute measure of the amount of enzyme expressed.

The results are shown in Table 3.2. Highest expression is seen in the liver, with kidney and testis reaching 20% of the liver value. Of the other extrahepatic tissues where message was detected (1-8% of liver values) the lung was qualitatively the highest.In both the protein based studies the kidney level of the enzyme was lower than the mRNA levels would predict, this may indicate translational control, which is discussed later.

Southern blot analysis shows the microsomal GST to be a single gene in the rat (DeJong *et al* 1988). In addition, the mRNA transcript size is 950 bp in all tissues. Although a second transcript of 550bp is sometimes seen it is derived form the use of a second polyadenylation site in the 3' end of the gene (DeJong *et al* 1988). The same pattern is observed when the full length cDNA is expressed in *Saccharomyces Cerevisiae* confirming the all the mRNA to be produced from the same gene(Chapter 4) Taken together these results make it unlikely that the lack of NEM activation of the protein in testis [from Morgenstern *et al* (1984)] is due to expression of an isoenzyme. DeJong also states that the RNA expression observed "would predict considerably higher activity from liver. The poor correlation between the relative levels of RNA expression here and the immunochemically quantitated protein in extrahepatic tissues [Morgenstern (1984)] is puzzling." As suggested before there may well be translational regulation in operation. In this respect the human microsomal GST shows some evidence that the 5' end of the gene can exert an effect on translation. At position -33 to -31bpupstream of the ATG there is an out of frame ATG, when translated this produces a hexapeptide which is terminated at position+20 to+22bp by a TAA (DeJong *et al* 1988). This arrangement would depress the initiation from the authentic ATG (Kozak 1984). No such observations have been made in the rat, but there is an 8bp repeat, AAGATTGA, at positions -23 to -17bp and at positions -12 to -5bp. However, no significance has been attached to these sequences.

In common with the cytosolic GSTs, the microsomal GST in the rat is predominantly located in the liver (Ketterer *et al* 1988), see Table 3.2. It is noteworthy that the human pattern of distribution is more even. It must be pointed out that this data is total CDNB activity, which does not

account for isoenzymes with low activity for this substrate, such as the theta class (Ketterer *et al* 1988). The microsomal GST has low activity for CDNB and a lower concentration than the cytosolic enzymes, and thus would not greatly contribute to these values. Reverse phase HPLC analysis of liver, lung, kidney and testis cytosol has been performed (Ketterer *et al* 1988). The profile of the isoenzymes varies greatly for instance the subunit 8 is highly expressed relative to other tissues in the lung. This isoenzyme has particularly high activity for 4-hydroxy-2,3-*trans* nonenal (4HNE) a toxic product of lipid peroxidation. Western blot analysis of extrahepatic tissues also demonstrated a particularly high concentration of YkYk (GST8-8) in the rat lung (Hayes and Mantle 1986).

Summary of results in 3.4.1.

By investigating the tissue distribution of the microsomal GST, some idea as to this protein's function may have been elucidated. In common with other drug metabolising enzymes, the highest level of expression is in the liver and, in addition, it shares the same hepatic location. The expression in the liver was a least tenfold higher than that observed in the extrahepatic tissues studied. The lung contained the highest content of microsomal GST protein after the liver.and this might signify the protective role of this enzyme against oxidative stress.

There is some indirect and direct evidence to suggest Morgenstern's microsomal GST is not the only form of GST present in the microsomes. The evidence comes form the activity measurements made in microsomes of extrahepatic tissues and also, other microsomal associated forms of GST have been described (Dierickx *et al* 1986, Asano *et al* 1990). Purification of these enzymes from extrahepatic tissues maybe important. Much discussion has been focused on the merits of a membrane location, that further investigations of the active state of the microsomal GST and/or the existence of other species warrants further investigation.

The next section approaches the endogenous regulation of the microsomal GST.

3.4.2.Developmental regulation of the mouse microsomal glutathione S-transferase.

The development of the microsomal GST expression in the mouse was followed from 2 weeks after birth into adulthood at 10 weeks. The onset of puberty in the mouse is considered to be between 4-6 weeks. Western blots of microsomes from liver kidney lung and testis using the microsomal GST antibody are shown in figures 3.3.

Two weeks after birth levels were detectable, but low in all four tissues. In the liver and the lung, there was a sharp increase at four weeks, with a further gradual increase from 8 to 10 weeks. The pattern is similar to that found in the kidney, but the increase is not so dramatic. A slight sexual dimorphism was observed after four weeks in the liver which showed the male levels being higher than females As with the rat the amount of protein in the kidney is low as compared with the liver. For the kidney, lung and testis microsomes ten times more protein was loaded in than liver. The testis differed from the other tissues studied in that there was no marked change between 2 and 10 weeks, the only difference being a slight drop in expression at 4 weeks.

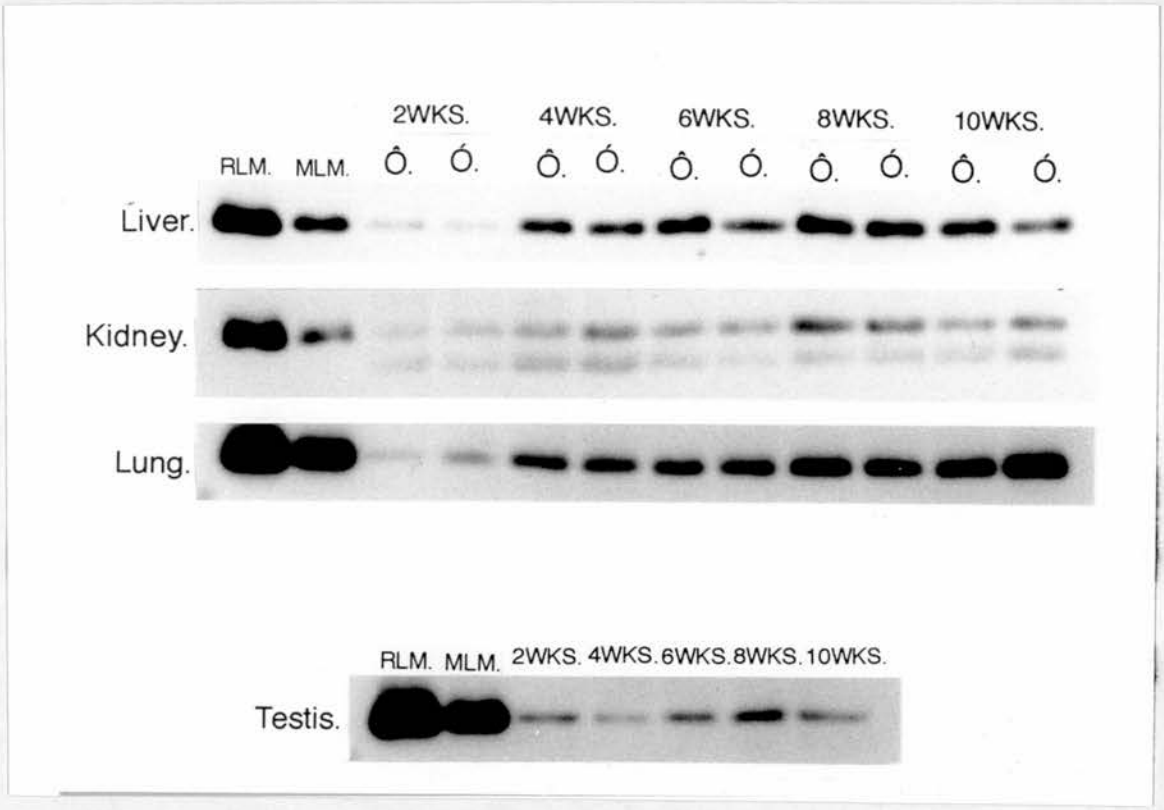
In the liver, lung and kidney development regulation of the mouse microsomal GST is observed. The pituitary gland has been implicated in playing a role in this regulation. To evaluate the extent of the involvement of pituitary gland, expression of the microsomal GST was investigated in hypophysectomized animals.

3.4.3. The role of the pituitary gland in the regulation of rat and mouse microsomal glutathione S-transferase.

The effect of hypophysectomy in conjunction with phenobarbital and dexamethasone treatment in the liver was examined in the mouse. In the rat only the effect of hypophysectomy on microsomal GST expression was investigated, as previous studies had shown that expression was unaffected by xenobiotics in this animal (Morgenstern *et al* 1982; DeJong *et al* 1988).

The results for the rat liver are shown in Figure 3.4. No difference

Figure 3.3. The developmental regulation of the mouse microsomal glutathione S-transferase.



MWM: molecular weight markers.

RLM: 10μg of rat liver microsomal protein loaded.

Ó: female.

Ô: male.

WKS: weeks.

3μg of protein of liver microsomes were loaded.

30μg of protein of kidney microsomes were loaded.

30μg of protein of lung microsomes were loaded.

37.5μg of protein of testis microsomes were loaded.

Figure 3.4. The role of the pituitary gland in the regulation of the rat liver microsomal glutathione S-transferase.

MWM: molecular weight markers.

RLM: 10μg of rat liver microsomal protein loaded.

Ó: female.

Ô: male.

3μg of protein of liver microsomes were loaded.

Con: control.

Hypox: hypophysectomised animals.

Sham: operated animals.

is seen between hypophysectomised rats and the control operated (sham) animals. A slight drop is observed between the control and the hypophysectomized/sham-operated animals. It may be concluded that the operation or some other experimental factor other than the pituitary has influenced expression. The overall interpretation suggests that the regulation of rat microsomal GST doesn't involve the pituitary, in the liver.

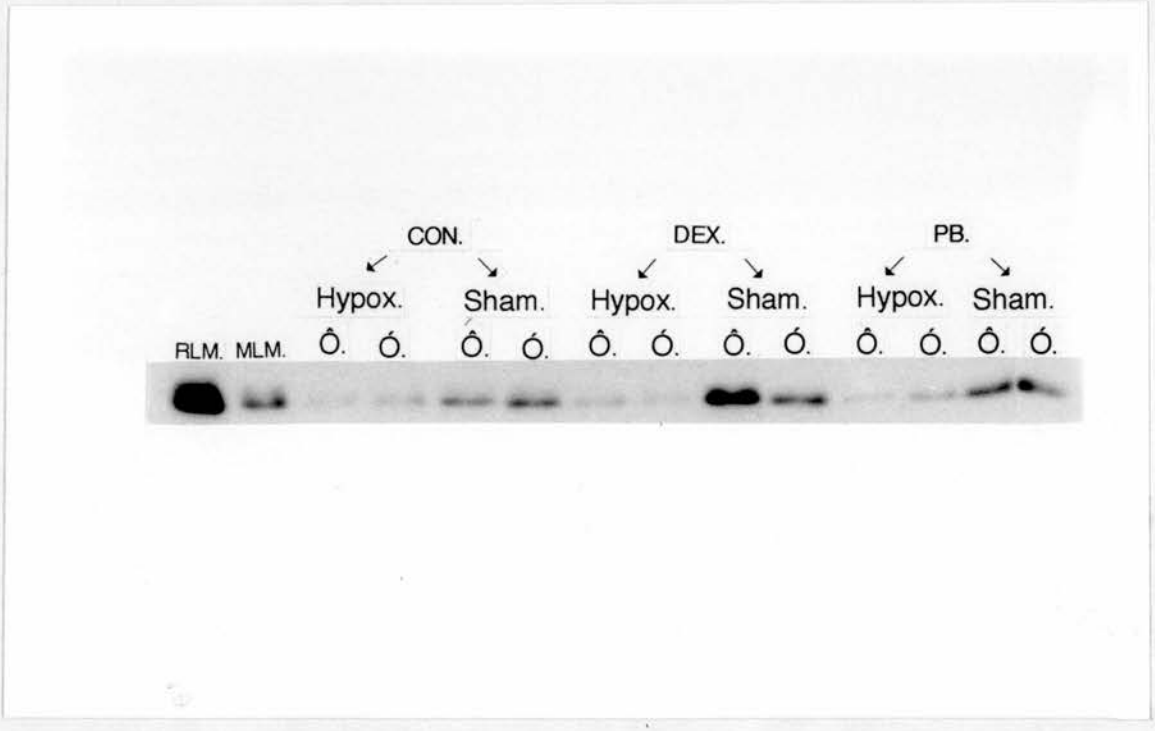
The data in the mouse, however, does suggest pituitary involvement (Figure 3.5). Hypophysectomy suppresses the expression of the microsomal GST in the liver. A slight induction is seen with dexamethasone in the male sham-operated mice, but was not observed in the hypophysectomised animals. Dexamethasone is a synthetic glucocorticoid, which inhibits growth in man and laboratory animals. In a recent study dexamethasone treatment was shown to alter the growth hormone secretion profile in man (Pralonge *et al* 1991). This result in the mouse may implicate a role for growth hormone in the regulation of microsomal GST expression. Phenobarbital and dexamethasone have been shown not to affect the rat microsomal GST expression. (DeJong *et al* 1988).

Further investigation was carried out at the transcriptional level. Northern blot analysis is shown in Figure 3.6. The mRNA levels for the control and dexamethasone treatments mirror the observed protein levels. The slight induction of the microsomal GST in the dexamethasone-treated male sham mice is again observed at the RNA level. However it is not as marked as the change observed at the protein level. When looking at the sham-operated male control mice, this also seems to be slightly raised with respect to the female sham operated control, having the effect of lessening the difference observed with dexamethasone treatment.

The observed suppression of the protein in the hypophysectomized animals is not observed in the phenobarbital treatment. Phenobarbital-treated sham-operated levels of microsomal GST are comparatively low compared with control/sham-operated animals, which may go some way to accounting for these discrepancies.

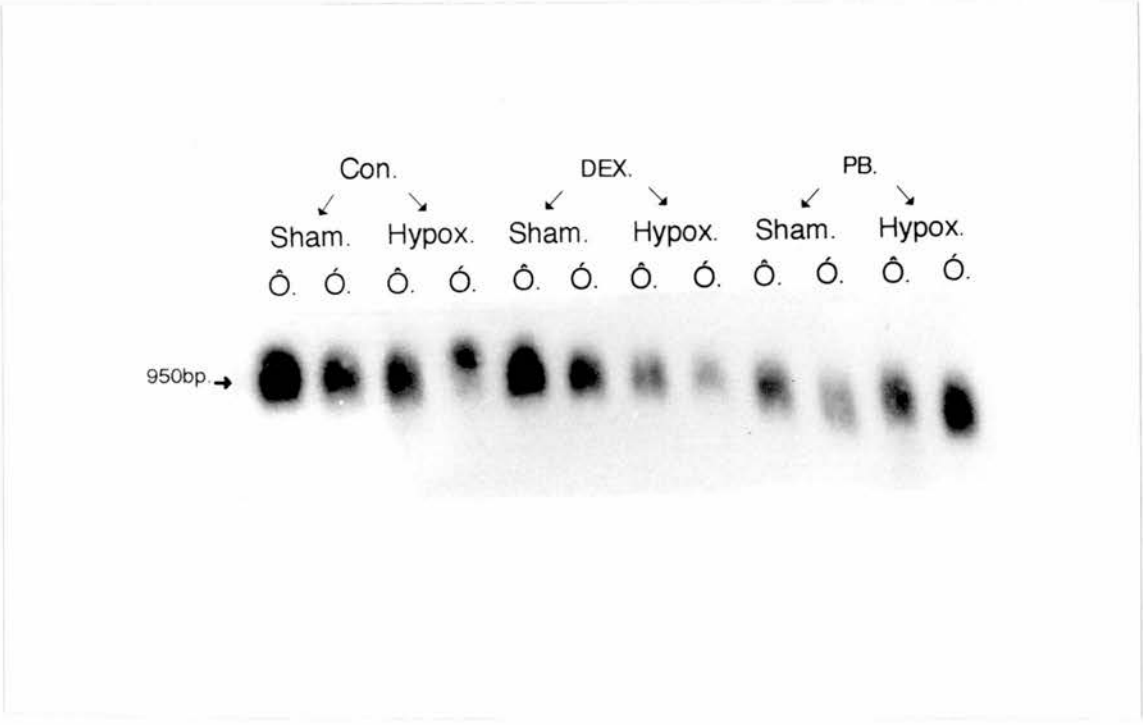
A number of other drug metabolising enzymes show pituitary regulation and suppression on hypophsectomy see Section 3.12 and

Figure 3.5. The role of the pituitary gland in the regulation of the mouse liver microsomal glutathione S-transferase by Western blot analysis.



RLM: 10μg of rat liver microsomal protein loaded.
MLM: 10μg of mouse liver microsomal protein loaded.
Ó: female.
Ô: male.
3μg of protein of liver microsomes were loaded.
Con: control.
Hypox: hypophysectomised animals.
Sham: operated animals.
DEX: dexamethasone treated animals.
PB: phenobarbital treated animals.

Figure 3.6. The role of the pituitary gland in the regulation of the mouse liver microsomal glutathione S-transferase by Northern blot analysis.



10μg of total RNA was loaded.
Ó: female.
Ô: male.
Con: control.
Hypox: hypophysectomised animals.
Sham: operated animals.
DEX: dexamethasone treated animals.
PB: phenobarbital treated animals.

3.13.

As discussed in section 3.1, growth hormone secretion is one of the major mediators of pituitary action on hepatic expression. The little mouse model as described in Section 3.34 is useful to study the effects of growth hormone.

3.4.4. The role of growth hormone in the regulation of the mouse liver microsomal glutathione S-transferases.

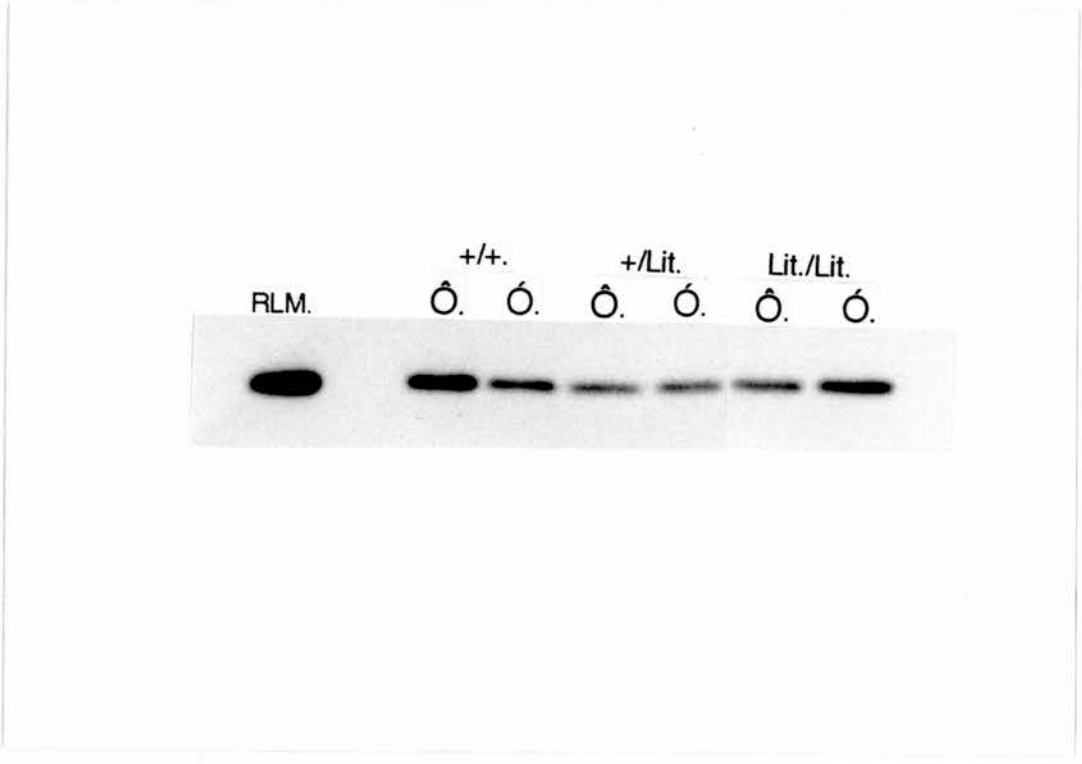
A number of hepatic proteins are thought to be regulated by the pituitary gland via the action growth hormone. Although the secretion of growth hormone is sexually differentiated, not all proteins governed by this pathway exhibit sexual dimorphism. (Zaphiropoulos *et al* 1989).

Figure 3.7 shows the Western blot analysis of homozygous (+/+) and (lit/lit) and heterozygous (lit/+)male and females. There is a slight dip in the heterozygous (lit/+) strains, which has been observed for other drug metabolising enzymes (C.J.Henderson Personal Communication). In the control for the little mouse model, homozygous (+/+), the sexual dimorphism appears more pronounced, however the differences are reversed in the absence of growth hormone (lit/lit). However from the results here it is not possible to determine the precise mechanism if growth hormone action.

Other techniques are available to study the effects of growth hormone, such as the neonatal treatment of animals with mono sodium glutamate, which causes almost complete disappearance of GHRF by destruction of the GHRF immmunoreactive bodies in the arcuate nucleus and GHRF immunoreactive fibres in the median emince (Pampori *et al* 1991). The pattern of growth hormone can then be administered artificially and the effect on expression of a particular protein assessed. Currently the molecular mechanisms of growth hormone regulation of P450 enzymes is been studied by the analysis of the 5' non-coding region (Mode *et al* 1992). Further analysis of the regulatory mechanisms involved in the control of the mouse microsomal GST by growth hormone would warrant similar studies.

From these results it would appear that the pattern of pituitary

Figure 3.7. The role of growth hormone in the regulation of the mouse liver microsomal glutathione S-transferase.



RLM: 10μg of rat liver microsomal protein loaded.
3μg of protein of liver microsomes were loaded.
Ó: female.
Ô: male.
+/+: control C57BL/6 mice.
+/lit.: heterozygous little mice.
lit/lit.: homozygous mice.

regulation of the mouse microsomal GST is mediated by growth hormone secretion The exact nature of this mechanism can not be determined from these results and requires further study.

3.5. Summary.

In common with other drug metabolising enzymes the microsomal GST is most highly expressed in the liver. Also the microsomal GST is located in other areas where endogenous oxidative attack may be prevalent, the adrenal glands and lung. However, when considering the role of this enzyme in conjugating an individual substrate the relative activity and concentration of other enzymes capable of the same reaction must be considered, namely the cytosolic GST's and other glutathione dependent peroxidases and examples have been discussed in Chapter 1.

In common with many other hepatic enzymes, the pituitary gland regulates microsomal GST expression, although only in the mouse. The pituitary gland may exert some effect via growth hormone as has been seen with other xenobiotic and steroid metabolising enzymes in the liver. Isolation of the gene and study of the 5' non-coding region will no doubt yield some interesting findings.

Chapter 4: The expression of the rat microsomal glutathione S-transferase in *Saccharomyces cerevisiae*.

4.1. Introduction.

4.1.1. Overview of the uses of recombinant expression systems in the study of drug metabolism.

The overview presented in this section is designed to serve Chapters 4,5 and 6. The specific nature of each chapter is discussed in more detail at the start of each chapter.

The use of *in vitro* enzyme assays to examine the function of a protein in a detoxification pathway has provided much useful information (Mannervik and Danielson 1988; Ketterer *et al* 1989). For example, the study of individual GSTs involved in the protection of cells against lipid peroxidation has revealed a spectrum of activities within the family of enzymes (Chapter 1.5). Proteins in the family with particularly high activities towards specific substrates are obvious candidates for the protective role against such compounds. However, other considerations apart from the enzyme activity have to be taken into account before the physiological role of the GSTs can be assigned. These include the regulation, tissue distribution and relative levels of expression (discussed in Chapter 3). In many cases, the GSH conjugation is not the only or rate limiting step in the fate of a substrate. For example, inhibition studies of the mercapturic acid pathway during the metabolism of hexachloro-1,3-butadiene have shown that the glutathione conjugates are not themselves responsible for the ultimate toxic effect, but this is produced by the action of β lyase on the cysteine conjugates (Anders *et al* 1988; Koob and Dekant 1991).

These examples point to the limits of *in vitro* studies and the need to develop *in vivo* models. In toxicology the whole animal model has been widely used in studying the metabolic fate of a compound, although much valuable information can be obtained the complexity of the system is a limiting factor in assessing the role of an individual enzyme.

An increasingly exploited approach, with the advent of

recombinant DNA technology, is to make use of heterologous expression systems (Simula *et al* 1993). Use of such systems in bacteria, yeast and mammalian cell culture has allowed the study of xenobiotic metabolising enzymes in a cellular environment. Unicellular organisms in particular are useful as each cell has the complete a complement of cellular machinery to survive, this allows the investigation of the role of cellular metabolism in the toxicity of a particular compound. Conversely it could be argued, that these systems are an oversimplification, such organisms may lack the tissue-specific pathways found in the multicellular state. This explains the organ-specific toxicities observed, for example the nephrotoxicity of the halogenated hydrocarbons such as HCBD. More complex systems using transgenic animals (Simula *et al* 1993), and *Drosophila Melanogaster* (Jowett *et al* 1992) are currently being developed.

In the 1970-1980s short-term tests (STT) for mutagens and carcinogens were employed in the toxicological world. These tests used micro-organisms as described above, to screen various chemicals.The most famous is probably the Ames test (Ames 1979), which involves exposing a His^- strain *Salmonella typhimurium* to the chemical and assessing its mutagenicity by determining the reversion frequency of the His^- mutation. However, the concordance with observed results in animal systems was poor only 60% (Tennant *et al* 1987). The limitations of such STT was explained by the failure of the micro-organisms own xenobiotic systems to activate potential mutagens. One solution was to add mammalian subcellular fractions, S9 fractions or microsomes (Lu and West 1986; Wolf *et al* 1986; de Montellano 1986). However activation of the compounds would take place outside the cell limiting access of reactive species to cellular targets, also addition subcellular fractions contain a number of different enzymes.

A number of investigators have expressed xenobiotic metabolising enzymes in recombinant systems and exploited the already existing mutagenicity tests, allowing the study of an individual enzyme and increases the sensitivity of the system towards the relevant substrates.

The potential of such systems are boundless, not only for the study of xenobiotic metabolism, but have been applied across the field of

biology. Site-directed mutagenesis (SDM) has allowed the investigation of catalysis and function. Recombinant expression has facilitated the purification of proteins where endogenous levels are low. Each cell type has its relative merits, for example the presence of the endoplasmic reticulum membrane in yeast has facilitated their use for studying P450s (reviewed by Guengerich *et al* 1991). The use of bacteria for the expression of membrane proteins has limitations, however some success has been achieved by altering or removing N-terminal sequences of P450 and P450 reductase (Larson *et al* 1991; Barnes *et al* 1991; Shen *et al* 1989).

The simplistic nature of yeast and bacteria has enabled detailed study of their transcription and translation machinery to be made. This depth of understanding has lead to the construction of a wide range of expression vectors, as a result stable expression of a number of proteins has been achieved. However the evolutionary distance between these organisms and higher eukaryotes has lead to problems in achieving successful expression. The explanations for failure of these systems are prevalent in the literature. There are marked differences in codon usage between organisms, which may be a limiting factor in translation. Differences in RNA processing; post-translational modification; and misreading of cellular targeting signals can also present potential problems. Expression in mammalian systems has been used to overcome these evolutionary differences, however the technology is not as advanced (Eastabrook *et al* 1991 with reference to P450). Recombinant expression is not an exact science nor probably that well understood. Despite strategies to overcome various problems results are often unexpected and example specific.

The relevance of the data obtained from the use of heterologous model system in the study of xenobiotic metabolism is always open to question. The manifestation of chemical insult on a cell depends on a number of factors. Expression of a foreign protein exerts its own stress on the cell and may induce changes which alter the cells sensitivity to a particular chemical. An example, is the expression of superoxide dismutase (SOD) in bacteria (Bloch and Ausubel 1986), when exposed to paraquat, a superoxide generator, the SOD expressing bacteria where

more sensitive to the chemical than the wild type. Further investigation revealed that the over expression of SOD interfered with the induction of the sox RS regulon, in particular the glucose-6-phosphate dehydrogenase. This reduced the ability of the SOD expressing bacteria to adapt to the presence of the paraquat, leading to a greater susceptibility to the compound (Liocher and Fridovich 1991).

4.1.2. The recombinant expression of the cytosolic glutathione S-transferases in the study of xenobiotic metabolism.

More than 25 years ago increased glutathione content, resulting in increased detoxification, was proposed as a mechanism of drug resistance in tumour cells (Hirono 1960). In more recent years studies have shown that drug refractive tumours and cell lines have elevated GST levels and this has been put forward as a resistance mechanism (reviewed by Hayes and Wolf 1988,1991).

This increase in expression is not confined to one isoenzyme, but varies according to cell type and the agent used. An ovarian adenocarcinoma cell lines were derived before and after the onset of resistance to cis-platinium, chlorambucil and 5-fluorouracil, the drug resistant cell lines showed a 3-fold increase in GST activity (Lewis *et al* 1988). Another popular approach has been to induce resistance in a cell line by exposure to drug, while maintaining a wild type population for comparison. A Walker 256 rat breast carcinoma cell line was made resistant to chlorambucil. The wild type cell line expressed mainly GST subunit 7, with trace amounts of subunits 1,2 and 4. In the resistant cell lines the α class subunit was raised, while the μ class subunit 4 was decreased (Clapper *et al* 1987). The human breast cell line MCF7, when made resistant to adriamycin shows a 25 fold increase in π class GST (Batist *et al* 1986). The MCF7 cell line shows very low intrinsic GST levels compared with other human cell lines. Further study of the adriamycin resistant MCF7 cell line revealed alterations in GST expression were not the only changes to have taken place: DNA repair

Table 4.1 The heterologous expression of the cytosolic glutathione S-transferases in the study of xenobiotic metabolism.

	Increase in resistance (fold).											
Cell line.	*S.Cerevisiae* [a,b]			COS7[c]		10T^{1}/2[c]		NIH-3T3[d]		MCF7[e]	MCF7[f]	
GST isoenzyme.	B1B1	π	Ya	Yb1	π	Ya	π	π1	π2	B1B1	B1B1	
Class.	α		α	μ		α				α	α	μ
Compound.												
Adriamycin.	3-16	2.3-10	1.3	nsd	1.3	-	-	1.8	3.0	nsd	nsd	nsd
Chlorambucil.	2-8	2-5.2	1.4	1.3	1.2	1.5-2.9	1.7	nsd	nsd	nsd	nsd	nsd
Melphalan.	-	-	1.3	1.1	1.1	-	1.5	nsd	nsd	nsd	nsd	nsd
Cis platinum.	-	-	1.4	1.5	1.3	-	-	nsd	nsd	nsd	nsd	nsd
Benzo(a)pyrene.	-	-	1.2	1.3	1.5	-	1.1-1.5	-	-	nsd	nsd	nsd
Ethacrynic acid.	-	-	-	-	-	-	-	4.3	7.9	2.1-3	1.7	2.0
BCNU.	nsd	nsd	-	-	-	-	-	-	-	-	-	-
tBuOOH.	-	-	-	-	-	-	-	-	-	-	nsd	nsd
CDNB.	2.3	-5.0	-	-	-	-	-	-	-	nsd	nsd	nsd
CuOOH.	2.0	-	-	-	-	-	-	-	-	-	-	-
L-PAM.	-	-	-	-	-	-	-	-	-	nsd	nsd	nsd

[a] Black et al. (1990) Biochem. J.**268** 309-315.
[b] Black (1989). Ph.D. Thesis.
[c] Pulkalski et al .(1990) PNAS **87** 2443-2447.
[d] Nakagawa et al. (1990) JBC **265** 4296-4301.
[e] Leyland-Jones (1991) Cancer Research **51** 587-594.
[f] Townsend et al (1991) Molecular Pharmacology **41** 230-236.

Abbreviations: BCNU: 1,3- bis(2-chloroethyl)-1-nitrosourea ;tBuOOH: tbutylhydroperoxide; CDNB: 1-chloro-2,4-dinitrobenzene; CuOOH: cumene hydroperoxide; L-PAM: Lphenyl-alanine mustard; T10^{1}/$_{2}$: CH3 T10^{1}/$_{2}$; nsd: no signficant difference; -: not studied.

capacity and levels of P-glycoprotein had been increased. Adriamycin toxicity has a number of effects within the cell (membrane damage; DNA lesions; oxidative stress; inhibition of topoisomerase II etc.) such different effects would inevitable elicit a multitude of responses.

Recombinant expression systems provide an excellent opportunity to artificially elevate GST levels and thus create a model system to assess the role of the GSTs in producing the drug resistant phenotype. Table 4.1 summarises the data from a number of such experiments and demonstrates that the outcome of the cytotoxicity to varies according to the system used. Most obvious is the difference between the yeast and mammalian systems. An increase in resistance to adriamycin was observed on expressing the human α class B_1B_1 and π class enzymes in yeast (3-16 fold and 2.3-10 fold respectively) and also to chlorambucil (2-8 fold and 2-5.2 fold respectively) (Black *et al* 1990). In mammalian cell lines, the resistance observed was generally less and dependent on the cell line used. The level of CDNB activity in yeast, expressing the cytosolic GSTs, was similar to that found in mammalian liver cytosol and was a 100-200 fold higher than the yeast endogenous activity (Black *et al* 1990) the increase in activity observed in the mammalian cells was smaller, this may explain the more pronounced effect observed in yeast. The increase in levels α GST observed in MCF7 cell lines was reported to be similar to that observed in rat cell lines made resistant to chlorambucil, however the resistance achieved was not in the same order (Townsend *et al* 1991).

P-glycoprotein and GST π in the MCF7 were increased in a cell line made resistant to adriamycin (Batist *et al* 1986). In addition both these two proteins where raised, again in MCF7 when selected for vincristine resistance (Whelan *et al* 1992). Based on these observations these proteins were transfected independently and simultaneously into cell lines. Alone or in conjunction with P-glycoprotein GST π failed to alter sensitivity to adriamycin, colchicine, cisplatinium or melphalan (Moscow *et al* 1989, Fairchild *et al* 1990). These observations question the involvement of the two proteins in conferring the drug resistant phenotype.

A feature of drug resistant tumours is cross-resistance to chemically dissimilar compounds. Such patterns of cross-resistance has been observed between agents such as melphalan, cis-platinum, nitrogen mustards, alkylating agents and mitomycin C (Wolf *et al* 1990). Again this feature is not entirely reflected in the expression experiments.

What conclusions can be drawn from the use of these models to study the role of the cytosolic GSTs in drug resistance? What does the variation between systems mean in terms of the validity of the results ? In general terms the resistance was low when compared with that achieved in drug resistant cell lines. In this light it is reasonable to suggest that other changes are required to manifest the drug resistant phenotype or GSH conjugation is not the determining step in the phenotypic out come. The literature continues to argue over the relevance of such studies and the accuracy of the model. However what is clear is that *in vitro* metabolism by an enzyme, does not necessarily have a significant affect when translated to the *in vivo* state.

4.1.3. The use of heterologous expression systems in the study of the function of the microsomal glutathione S-transferase.

The microsomal GST is typically characterised by lower activities to substrates than cytosolic forms in *in vitro* studies. However, the microsomal GST has been implicated in having an important role, by virtue of its membrane location, in protection against peroxidative attack and metabolism of compounds generated in the membrane location. Expression in a recombinant system would allow the study of the enzyme in this respect. Regulation of this enzyme occurs via covalent modification of the protein and site-directed mutagenesis (SDM) offers a powerful means to study this phenomenon. The tyrosine 7 found in the cytosolic enzymes is conserved due to its central importance in catalysis, but this residue does not exist in an equivalent position in the microsomal enzyme. SDM would allow the investigation of catalytically important residues in the enzyme, which would lead to the understanding the catalytic mechanism. However sufficient measurable activity needs to be

achieved in order to be able to observe changes in kinetic parameters.

4.2 Expression of the rat microsomal glutathione S-transferase in *S. cerevisiae.*

4.2.1. Introduction.

The expression of any foreign gene in a recombinant system does not automatically guarantee a high level of expression of the corresponding protein. Protein expression is a complex multi-step process and problems may arise from transcription through to the stability of the protein product.

Reviews on yeast expression describe the available vectors for expression, but the merits are more difficult to assess (Romanos *et al* 1992). As is often the case the optimum conditions are example specific, and these can only be achieved by trial and error. Although the protein machinery of yeast has been well studied no precise rules apply to foreign protein expression and therefore, each case needs to be treated individually. However recombinant yeast technology has been in operation for a number of years and the literature is full of methods employed to optimise particular systems, which is accompanied by the individual researchers dogma.

In general, the recombinant expression of membrane bound proteins is less successful than the expression of cytosolic proteins. This is thought to be due to the higher stability of the latter. The study of N terminal sequences have shown typical membrane sequences to be more susceptible to turn over by the ubiquitin pathway- N terminal Rule (reviewed by Wilkinson 1991). Although the general process of membrane insertion is conserved there are subtle differences in signal targeting sequences. For example soluble proteins of the mammalian endoplasmic reticulum have a common tetrapeptide KDEL (Munro and Pehlam 1987), but in *S. cerevisiae* the sequences is HDEL (Pelham 1990). As a matter of interest this consensus varies amongst species of yeast, but they all have the core sequence XDEL (Pelham 1990). Also worth considering are the differences in lipid composition between organisms. Some membrane proteins have shown to be toxic when expressed in yeast, for example, *E.Coli* Omp A protein (Janawicz *et al*

1982), IV heamaglutin (Jabbar *et al* 1985), polyoama virus middle T antigen (Belsham *et al* 1986). This may be due to to non specific insertion or disruption of the yeast membrane.

However no hard and fast rule exists to cover membrane protein expression. Wide ranges of expression levels can be seen amongst families of membrane proteins for example the P450 enzymes (reviewed by Guengerich *et al* 1991), therefore other factors must come into play to contribute to the final level of expression.

Many factors affect the rate of synthesis of proteins, but the final yield must be equally affected by the rate of degradation. In this respect examples of high levels of protein expression have come from inherently stable proteins. For example superoxide dismutase (Halwell *et al* 1987), Hepatitis B core antigen (Kiniskern *et al* 1986) and *Schitsosomal* GST (Loison *et al* 1989) are all highly expressed in yeast. In fact the mammalian cytosolic GSTs are highly expressed in yeast (Black *et al* 1990), these proteins show a high degree of homology amongst species (Mannervik and Danielson 1988).

4.3. Aims of the chapter.

The yeast *S. cerevisiae* possesses an endoplasmic reticulum and mitochondria, making it suitable for the expression of the microsomal GST, which is located in these membranes in mammalian cells (Morgenstern *et al* 1984). Heterologous expression in this system would create a model for the study of xenobiotic metabolism by this protein. The regulation of the enzymes activity by post-translational modification could be usefully investigated by SDM.

This chapter describes the expression of the microsomal GST in *S. cerevisiae*, so that the role of this enzyme can be assessed in the metabolism of putative xenobiotic substrates. The chapter also goes on to describe measures taken to improve the system. The problems encountered with the setting up these types of systems is often omitted from the literature.

4.4. Results and discussion.

4.4.1. Expression of the rat microsomal glutathione S-transferase in *S. cerevisiae* using different expression vectors.

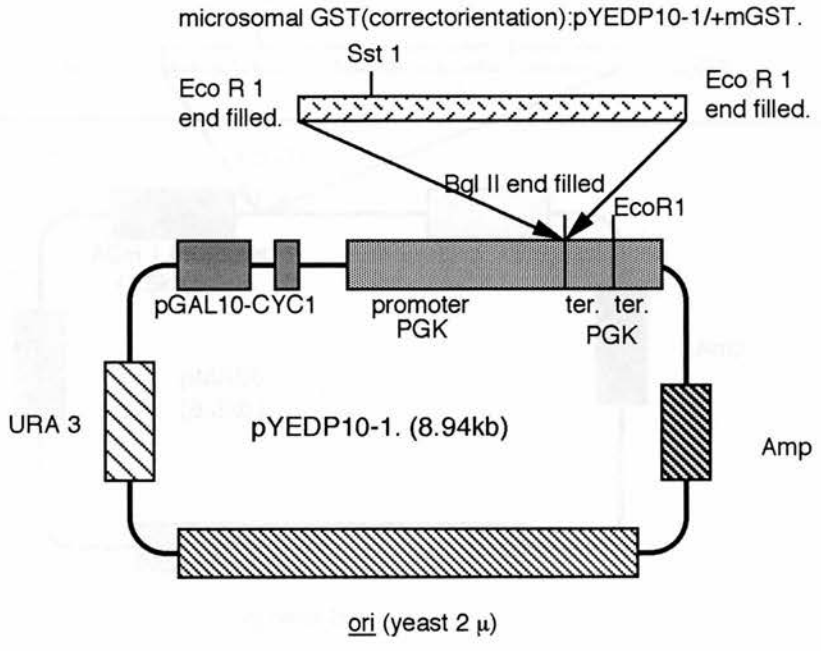
All the vectors employed were based on the commonly used two micron system (2μ). This is an endogeneous plasmid of 6.3 kb present in most *Saccharomyces* strains at about 100 copies per haploid genome. Vectors based on the 2μ plasmid will be represented at similar levels in the cell and replicate accordingly. The initial choice of promoters for yeast expression vectors where from the highly expressed glycolytic enzymes. All the vectors used here contain such promoters and are illustrated in figure 4.1, which also shows the cloning strategy employed.

The PGK promoter is a inducible promoter, which is regulated by the addition of glucose to the medium. This was the most powerful promoter used in the study. It consists of an upstream activating sequence (UAS) from -473 to -422 bp from the start site of the PGK gene (Kingsman *et al* 1990) Addition of 20% glucose to the medium can result in a 20 fold induction of mRNA, which can comprise of 5% of the total mRNA in the cell. Heterologus protein expression under the control of this promoter can be up to 1-5% of total cellular protein. The PGK promoter was present in pYEDP10-1 (Cullin and Pompon 1988) and was obtained from pMA 91 (Mellor *et al* 1983).

The GAL 10-CYC1 promoter present in pYEDP1/8-2 (Cullin and Pompon 1988) was obtained from pLGSD5 (Guarente *et al* 1982). The GAL 10 promoter is very tightly regulatable by 2% galactose in the media, but relatively weak in comparison to the PGK promoter.

The other two vectors pMA 56 (Ammerer *et al* 1983) and pVT100U (Vernet *et al* 1987) both contain the constitutive ADH 1 promoter. However, amongst other things, the vectors contain different terminators. pMA 56 contains 3' regions from FLP and pVT100U contains the appropriate sequences from the ADH gene. In all these constructs the 3' sequences of the rat microsomal GST were present.

Figure 4.1 Yeast expression vectors.



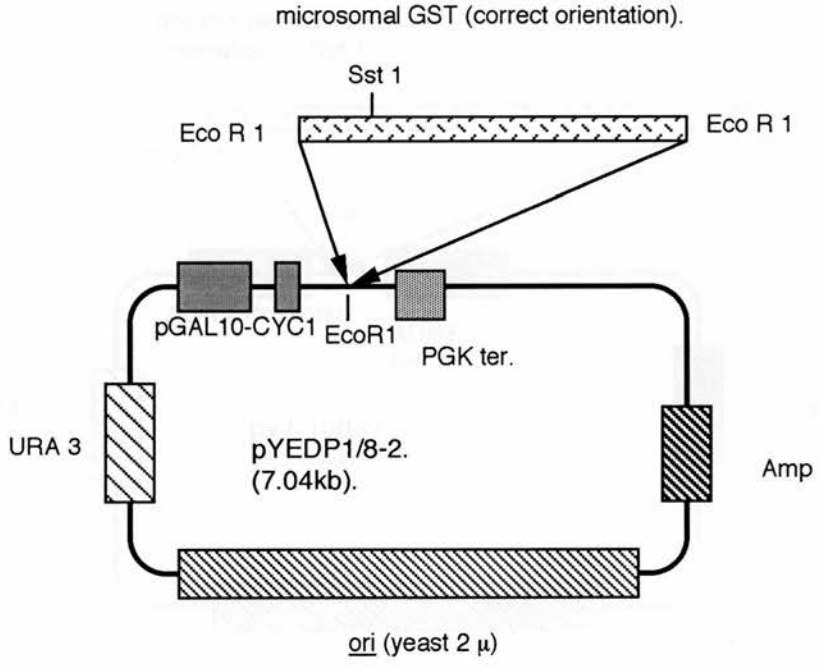
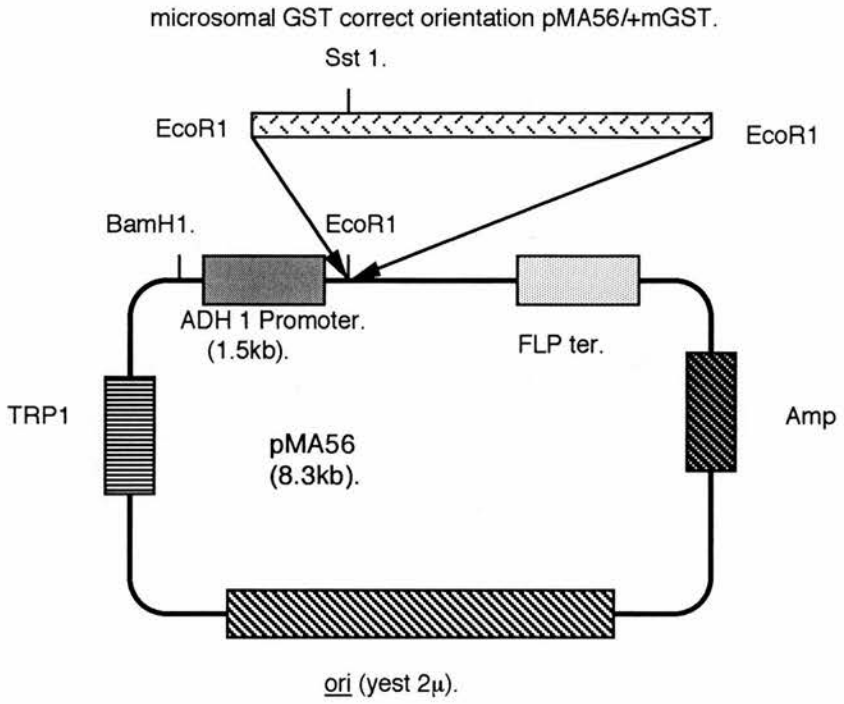
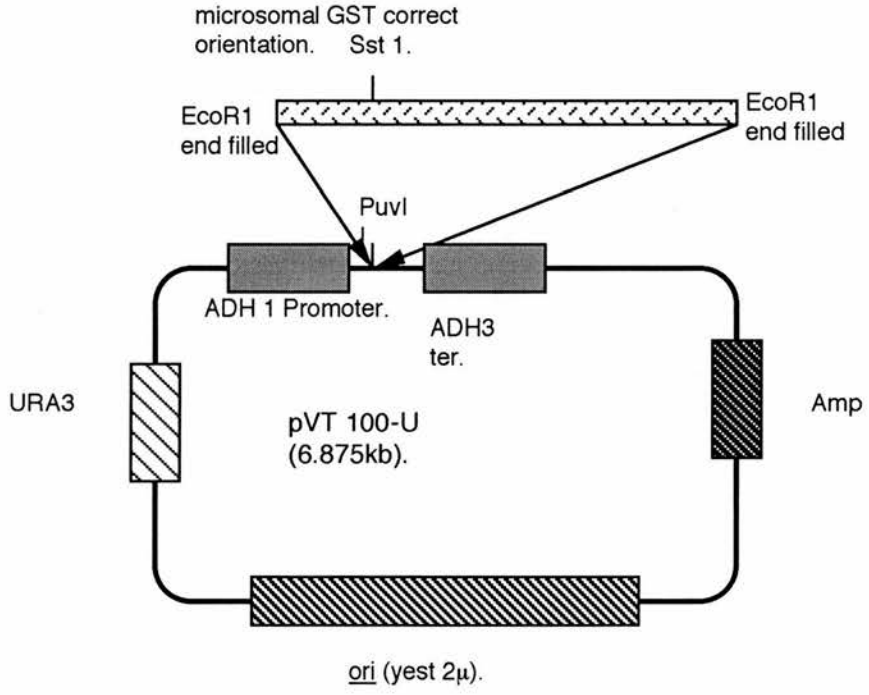


Figure 4.1 (continued) Yeast expression vectors.





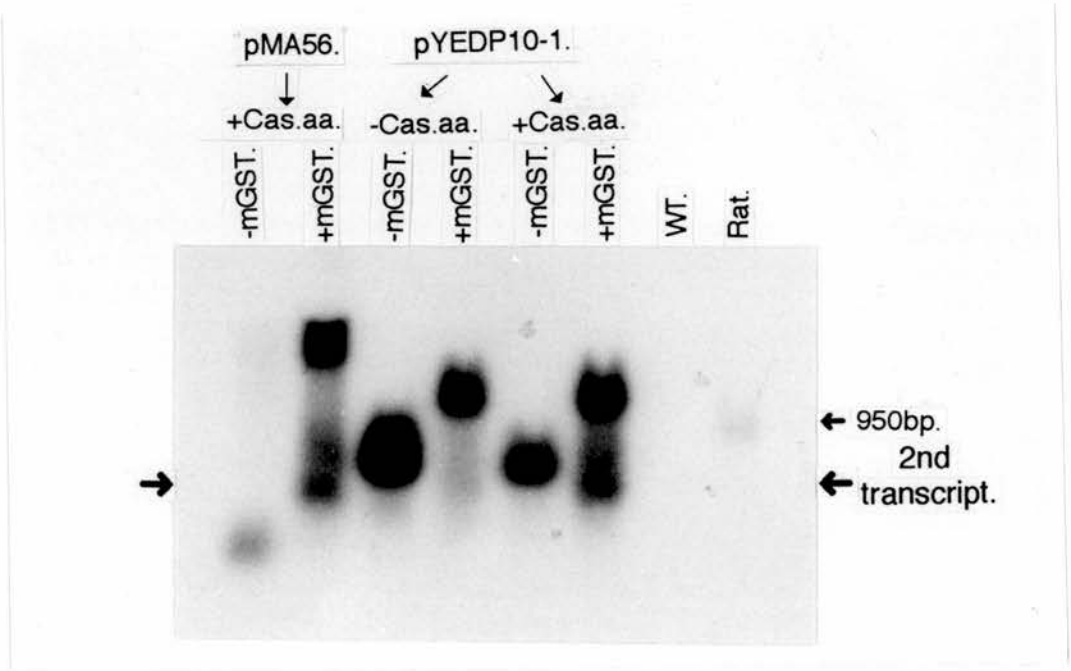
All the vectors had suitable selectable markers to allow the addition of cas amino acids to the medium, which is described in Section 2.1.8. In an enriched media the cells will grow faster and the general well being is enhanced. However, expression was only achieved from pYEDP10-1 and pMA 56 (data not shown). Yeast strains KY118 and W3031B were transformed with the pMA 56 and pYEDP10-1 derived constructs, respectively, as described in Section 2.1.10.

Figure 4.2 shows the Northern blot analysis of RNA prepared from the two successful expression constructs, probed with the microsomal GST cDNA. The level of microsomal GST mRNA present in the yeast transformed with the two expression constructs is considerably higher (per total RNA) than that found in rat liver. The level of expression of rat microsomal GST mRNA is slightly higher under the control of the PGK promoter than the under the ADH promoter. The mRNA from the pMA 56 vector appeared to be more smeared indicating a lower stability.

The 3' end of the rat microsomal GST contains two polyadenylation (polyA) sites (AATAAA) at position +557 to +565bp and +864 to +870bp. Analysis of RNA in the rat revealed a transcript size of 950bp, however in some tissues there was a faint transcript of approximately 550bp (DeJong *et al* 1988). On close examination of the Northern blot (Figure 4.2) a second faint transcript is observed, this is only observed in the the pMA 56/+mGST and pYEDP10-1/+mGST (+ cas amino acids), ie the constructs containing the cDNA in the correct orientation. In fact there is slightly more RNA in the cas amino acid containing media. Contrary to earlier ideas, it appears that yeast mRNAs follow the same pattern of termination, processing and polyadenylation as higher eukaryotes. However in yeast these processes are tightly coupled and occur within a shorter distance of the 3' end (Butler *et al* 1990).

Protein extracts were prepared from cultures of the two transformants as described in Section 2.4.1a. The proteins were separated on SDS-PAGE and subjected to Western blot analysis (Section 2.7.1 and 2.7.4, respectively). Figure 4.3 shows the Western blot probed with the anti-human microsomal GST antibody. The expression of the rat microsomal GST was greatest under the control of the PGK

Figure 4.2. Northern blot analysis of yeast transformed with expression constructs containing the rat microsomal glutathione S-transferase.



10 μg of total RNA loaded.

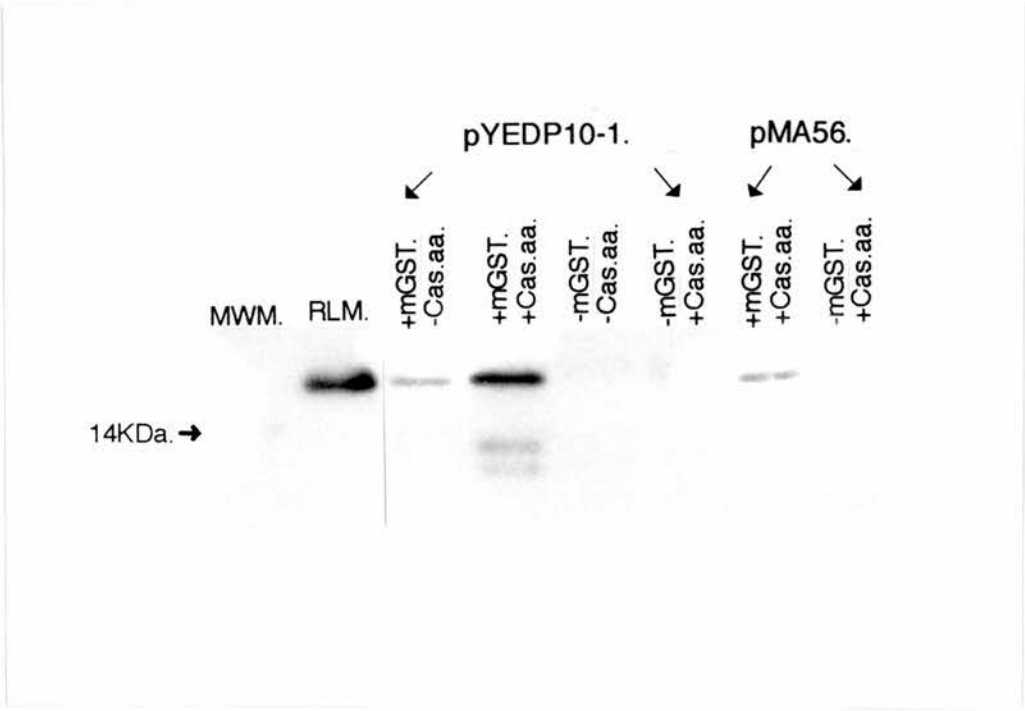
+mGST: cDNA in the correct orientation for expression in the vector.

-mGST: cDNA in the reverse orientation for expression in the vector.

Cas aa: growth media supplemented with Cas amino acids as described in Section 2.1.8.

WT.: wild type yeast.

Figure 4.3. Western blot analysis of yeast transformed with expression constructs containing the rat microsomal glutathione S-transferase.



RLM: 5μg of rat liver microsomal protein loaded.

50μg of yeast cell protein extract loaded.

+mGST: cDNA in the correct orientation for expression in the vector.

-mGST: cDNA in the reverse orientation for expression in the vector.

Cas aa: growth media supplemented with Cas amino acids as described in Section 2.1.8.

promoter. However the levels of protein were considerably lower than those seen in rat liver, inspite of the higher levels of mRNA levels.

Addition of cas amino acids to the medium increased the expression of protein from pYEDP10-1. Expression of the mouse P450 P1 from this vector (Cullin and Pompon 1988) was increased by changing the media to complete yeast media YPD in the last two growth cycles (D.Pompon personal communication). It is not unreasonable to expect enrichment of the media with amino acids will enhance protein expression and general well being of the cells. However, the increased expression is accompanied by an increased in degradation of the protein. Although pMA 56 and pVT100U contain the same promoter, pVT100U failed to express the microsomal GST. It would appear there are other factors within the make up of the vector are important.

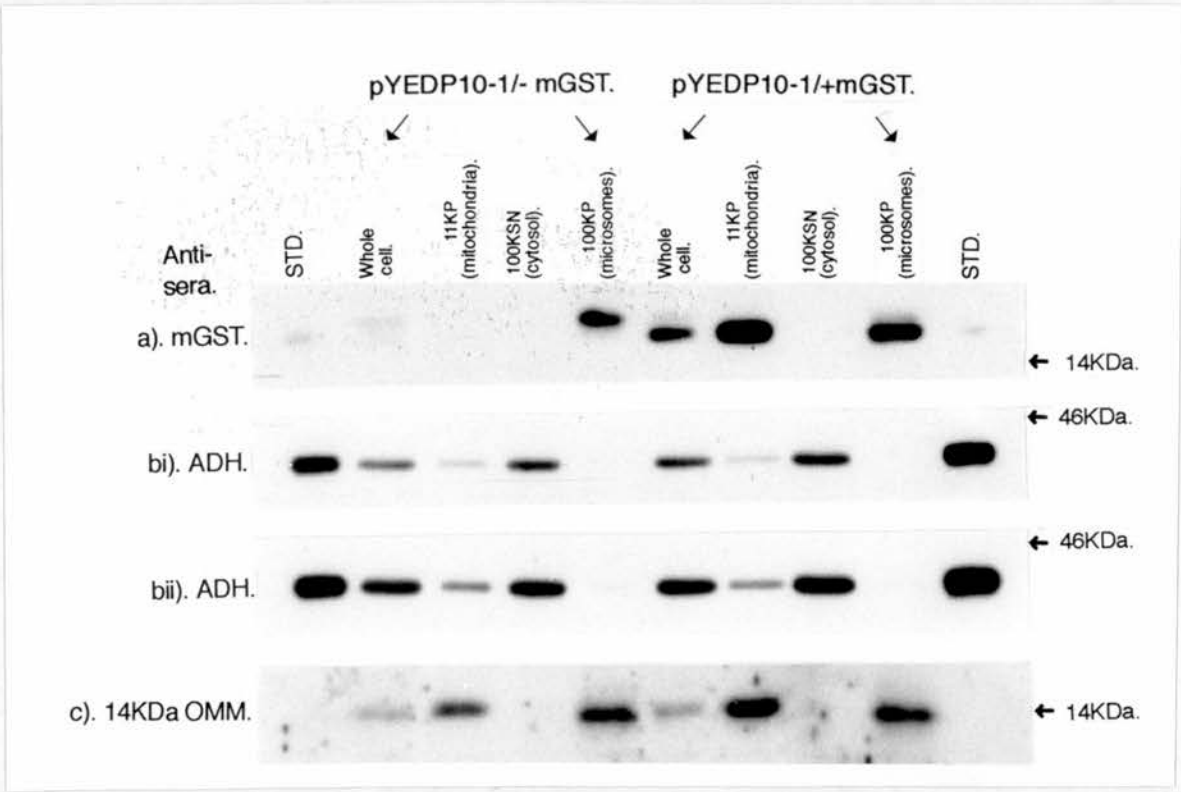
Four different constructs were made and only two produced protein. This indicates that the success in achieving expression depends on the individual construct. All future experiments were conducted using the pYEDP10-1 derived constructs, unless otherwise stated.

4.4.2. Expression of the rat microsomal glutathione S-transferase protein and subcellular localisation.

Subcellular fractionation were prepared from the yeast and then subjected to Western blot analysis, using the anti sera prepared against the recombinant microsomal GST purified from *E.coli* (described in section 2.75). Figure 4.4a demonstrates that the microsomal GST is located in the whole cell, mitochondria and microsomal fractions, this is in agreement with the observations in native protein in rat liver (Morgenstern *et al* 1984).

In order to asses the purity of the subcellular fractions prepared in the yeast, marker enzymes were used as follows. Figure 4.4bi shows the same fractions probed with anti-sera raised against the yeast alcohol dehydrogenase (ADH), this was the cytosolic isoenzyme. The majority of the ADH is located in the cytosol. However, some is found in the mitochondria fraction, this fraction received one wash during preparation and on a longer exposure of the autorad (Figure 4.4bii). Trace

Figure 4.4. Subcellular localisation of the rat microsomal glutathione S-transferase expressed in yeast.



pYEDP10-1/+mGST: pYEDP10-1 containing the rat microsomal GST in the correct orientation.

pYEDP10-1/+mGST: pYEDP10-1 containing the rat microsomal GST in the reverse orientation.

a) mGST: anti-rec. microsomal GST antisera.

50μg of yeast subcellular protein extracts loaded.

Standard: 5μg of rat liver microsomal (left hand side) mitochondrial (right hand side) loaded.

b) ADH: yeast alcohol dehydrogenase I antisera.

10μg of yeast subcellular protein extracts loaded.

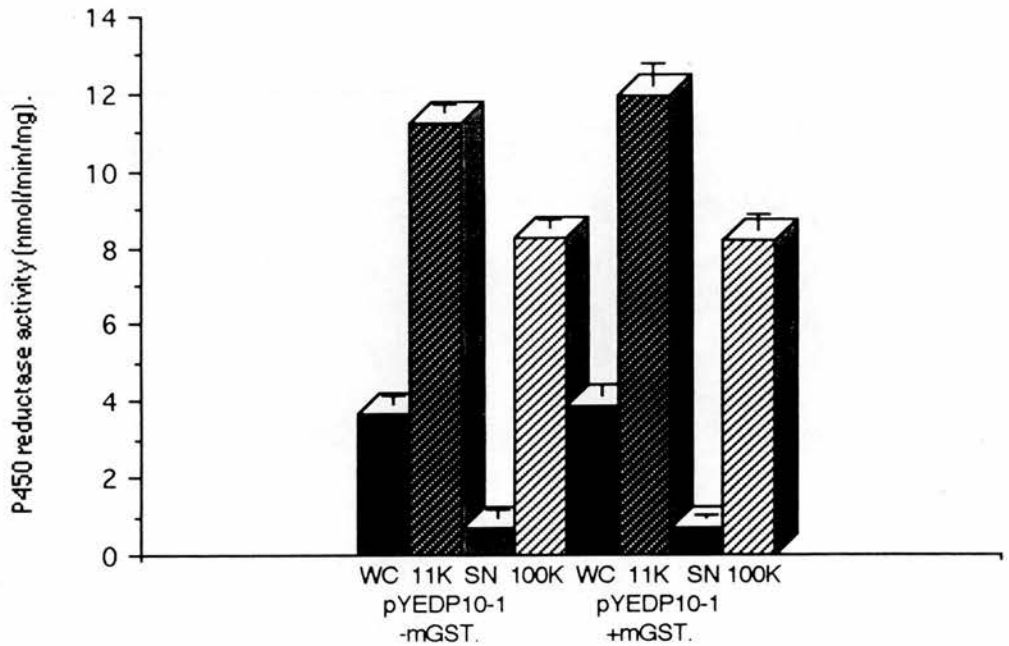
Standard: 1μg of purified yeast ADH (Sigma) loaded.

c) 14KDa OMM: yeast 14KDa outer mitochondrial antisera.

20μg of yeast subcellular protein extracts loaded.

Standard: none available (band identified due to molecular weight).

Figure 4.4d P450 reductase activity in subcellular fractions.



contamination of the microsomal fraction can be seen, but this fraction received three washes before resupention in the final sucrose buffer.

The 14KDa outer mitochondrial membrane (OMM) protein is found only in the outer mitochondria membrane and never in the endoplasmic reticulum, and it has been shown to be involved in porin structure (Riezman *et al* 1983). In the original paper describing the determination of the cellular localisation of 14KDa OMM sucrose gradients were employed to prepare subcellular fractions (Riezman *et al* 1983). Density centrifugation resolves the membrane fractions according to lipid composition, where as the differential centrifugation, used here, separates membranes vesicles due to density. Figure 4.4c shows the Western blot analysis of the subcellular fractions probed with antisera raised against the yeast 14KDa OMM (generous gift from G.Schatz). As can be seen from the blot the 14KDa OMM is present in the mitochondria and microsomal fractions. This is presumably due to the disruption of the fragile mitochondria membrane during preparation, which employed the French press.

In order to determine the purity of the microsomal fractions P450 reductase activity was determined using the spectrophotometric assay (Section 2.6.2). The reductase activity (not necessarily entirely P450 reductase) was found in both the mitochondria and microsomal fractions. This reflects the results with the 14KDa OMM antisera, and demonstrates that these fractions have not been resolved. Hence, it would be more accurate to term both the 11000g and 100 000g fractions as membrane fractions. In future experiments only the 100 000g pellet is considered and is termed as the membrane fraction. Figure 4.4d shows the P450 reductase activity in the whole cell, cytosol and membrane fraction, as can be seen the membrane fraction shows enrichment for the reductase activity. Another important observation is the levels of P450 reductase is the same in both control and expressing yeast, which indicates the uniformity of the fractionation procedure.

Electron microscopy would allow the viewing of the intact cell and give a more accurate determination of the subcellular distribution of the enzyme. However, the process is not with out artifacts and immunopure antisera would be required to carry out these experiments, which was not

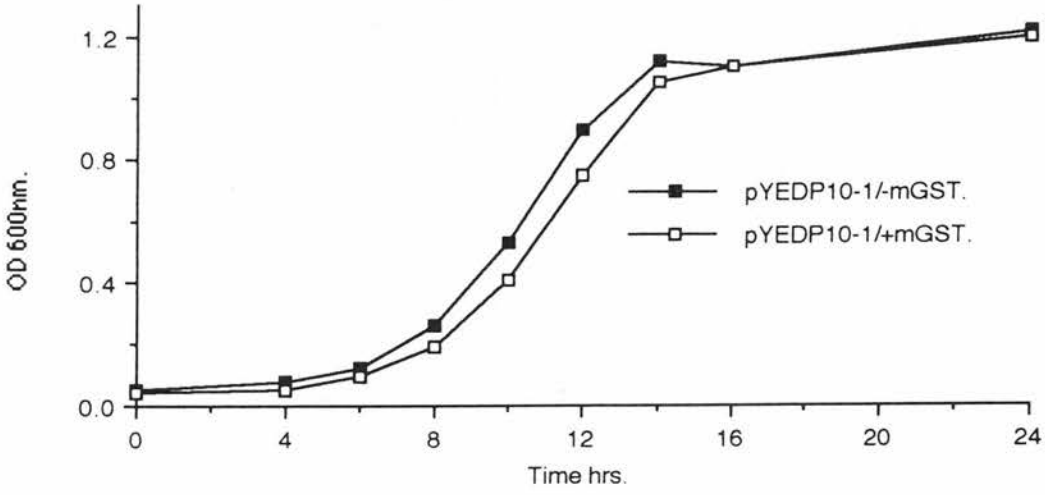
available at the time.

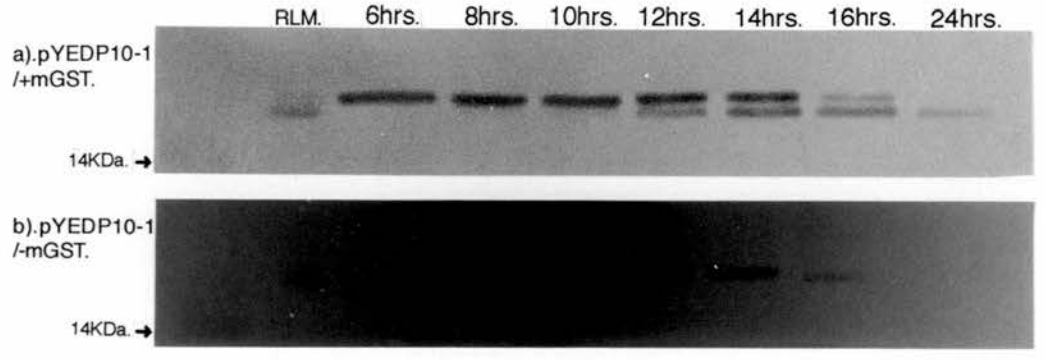
In summary of this section the microsomal GST is localised in the membrane fractions. It has been reported that the microsomal GST is found at 5% of mitochondria protein and 3% of microsomal protein (Morgenstern 1984). The preparation of the subcellular fractions in yeast, however, did not allow the separation of these two subcellular organelles.

4.4.3. The dependence of expression of the microsomal glutathione S-transferase, under control of the PGK promoter, on growth phase.

Maximum levels of heterologous expression depend on a number of factors, such as growth phase. Figure 4.5c shows the growth curves for expressing and control yeast together with Western blot analysis of whole cell extracts taken at different time points in the growth phase. Yeast were transformed with pYEDP10-1/+mGST and pYEDP10-1/-mGST and the results are shown in figures 4.5a and 4.5b respectively. The maximum expression of the microsomal GST, in this system, is during late log and stationery phase.

Figure 4.5 The expression of the rat microsomal GST in yeast during growth phase.





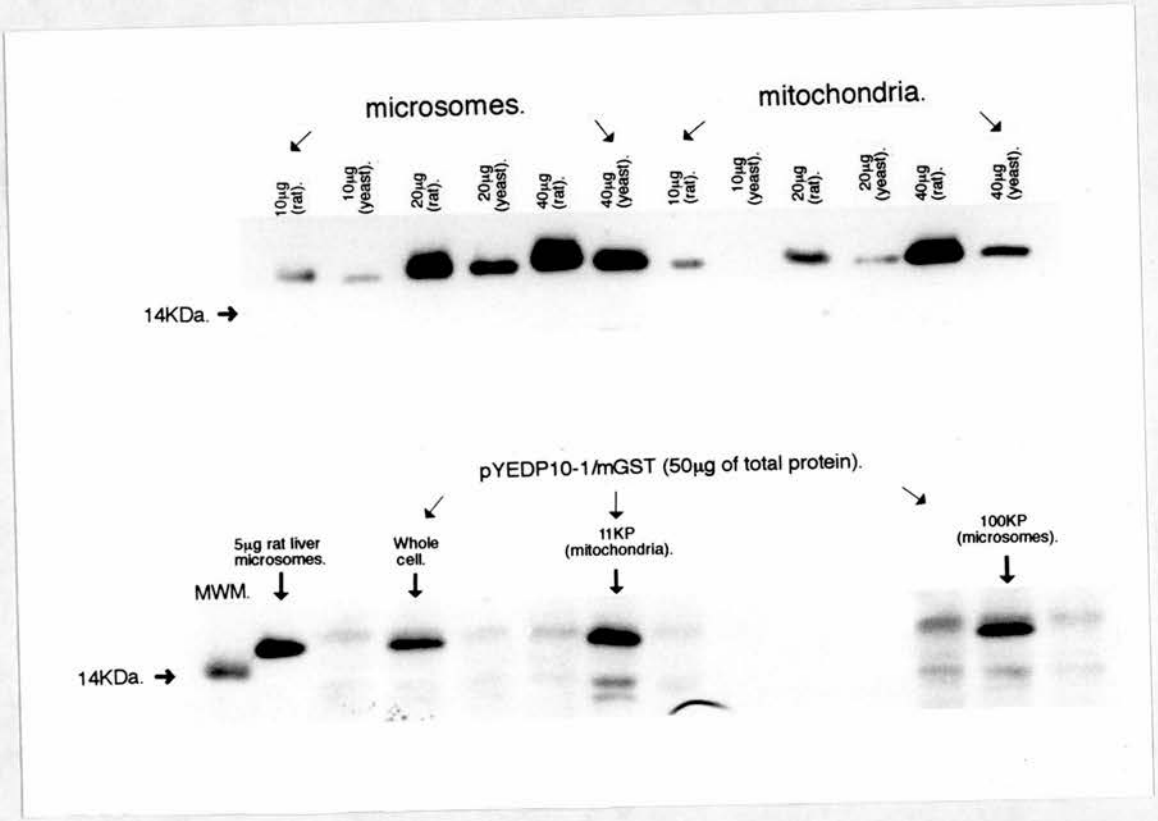
RLM: 5μg of rat liver microsomal protein loaded.

50μg of yeast cell protein extract loaded.

4.4.4. Estimation of the levels of rat microsomal glutathione S-transferase protein expression in yeast.

In order to make a quantitative assessment of the levels of protein expression, 10, 20 and 40 μgs rat and yeast membranes were loaded at onto 12% SDS PAGE gel. After transfer to nitrocellulose the blot was probed with the anti-rec. microsomal GST antibody and the bands were scanned with a Joyce-Lobell densitometre (Figure 4.6a). However the results were not consistent across the protein range described. A number of explanations can be offered, such as the nonlinear nature of both protein transfer to nitrocellulose and autoradiography film. Also it can be seen from the blots the native rat microsomal GST band is more diffuse than the recombinant protein in the yeast. This may be due to the higher density of bands found in yeast samples in this region compressing the recombinant rat microsomal GST band. The densitometre calculates the integral value from the width and height of the peak from the scanned band, a diffuse band will give a disproportionately large value. A second autorad (Figure 4.6b) was also scanned, where a tenth of the rat protein was loaded in order to give bands of a similar intensity, and this blot was probed with the anti-human microsomal GST. The results of both these experiments suggest the expression in the yeast membranes is between 10% and 50% of that in the rat microsomes.

Figure 4.6. Estimation of the levels of the rat microsomal glutathione S-transferase expression in yeast.



MWM: molecular weight markers

4.4.5. Assessment of CDNB activity in yeast expressing the rat microsomal glutathione S-transferase.

The CDNB activity of the recombinant microsomal GST was considerably lower than expected, when considering the level of protein expressed. The value measured in the yeast expressing the recombinant protein was 0.4 nmol/min/mg in this experiment, although values up to 0.8 nmol/min/mg were measured. Figure 4.7a shows the CDNB activities and figure 4.7b shows P450 reductase activities measured in yeast subcellar fractions. The CDNB activity is only significantly raised in the membrane fraction of the yeast expressing the rat microsomal protein, this trend was observed on at least three independent experiments. The P450 reductase measurements on the other hand are the same in control and yeast in this organelle. This suggests that the microsomal differences in CDNB activity are attributable to the expression of the microsomal GST.

CDNB is a poor substrate for the microsomal GST having a specific activity an order of magnitude lower than most cytosolic enzymes..This makes accurate determination of low values hard, however CDNB was the only substrate available.

The N-ethylmaleimide (NEM) treatment of the microsomal GST causes increase of the enzyme activity (Morgenstern and DePierre 1983), while decreasing the cytosolic GST activity (Morgenstern *et al* 1983b) On treatment of the yeast membrane preparations with NEM the difference between the control and expressing samples was slightly increased (Figure 4.7c), but not in the order of magnitude observed apon NEM treatment of rat liver microsomes.

The microsomal protein is thought to exist as a trimer (Morgenstern *et al* 1982). Recent evidence to suggest change in oligomeric composition maybe a means of modulating activity (Anyia and Anders 1992; Boyer *et al* 1986). Heat generated during the preparation of yeast membrane fractions may have been sufficient to disrupt the fragile oligomeric structure of this enzyme. It is of course it is arguable that the microsomal GST does not insert correctly in the yeast membrane, and hence not all the protein is active.

Figure 4.7a CDNB activity.

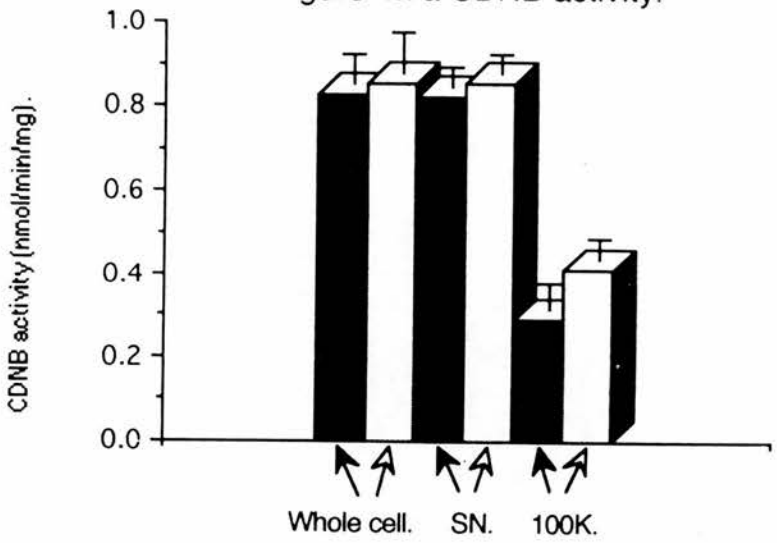


Figure 4.7b P450 reductase activity

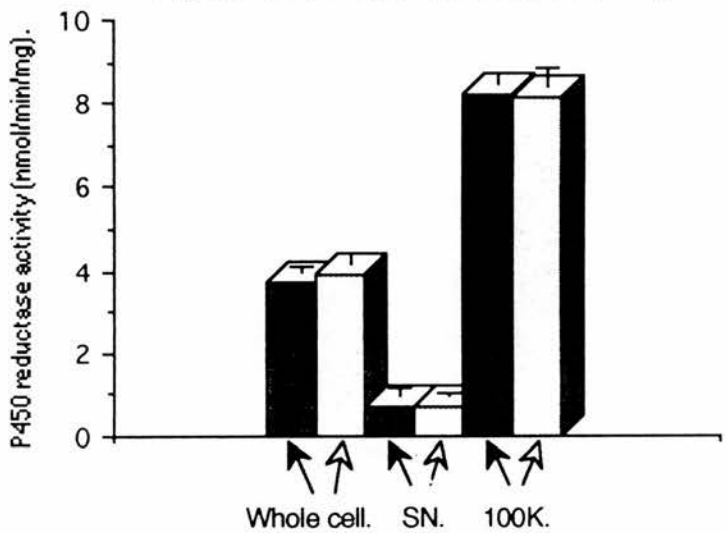
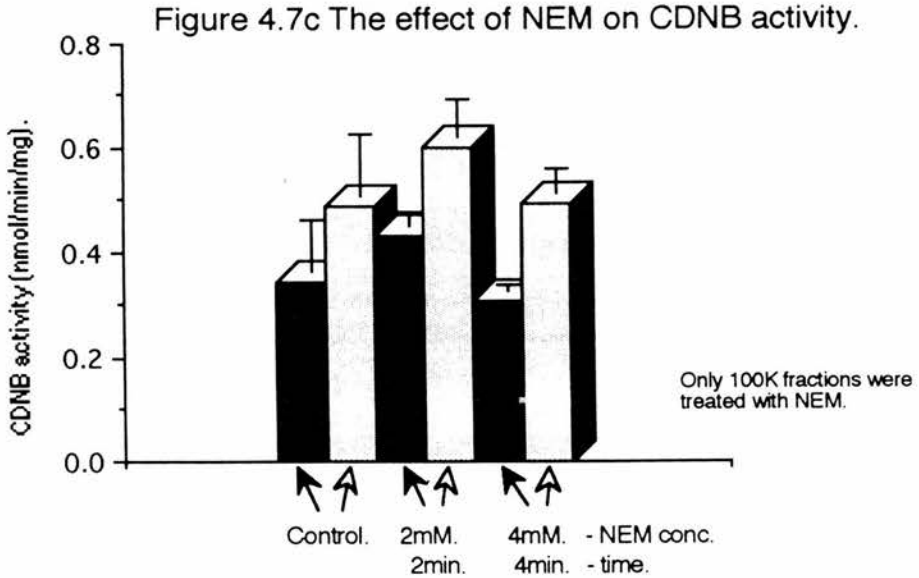


Figure 4.7c The effect of NEM on CDNB activity.



Abbreviations: SN = 100K supernatant (cytosol).
100K = 100K pellet (membrane fraction).

→■ pYEDP10-1/-mGST.

→□ pYEDP10-1/+mGST.

Another possibility is that the microsomal GST was damaged on preparation of the extracts. Reports suggest that the microsomal GST activity is sensitive to temperature change and also that activity and NEM inducibility is lost upon freezing (Morgenstern *et al* 1980). The instability of membrane proteins associated with change in temperature has often been attributed to disruption of the lipid bilayer, which results in loss of integrity and structure. In an attempt to stabilise the protein GSH was added to the buffers, not only is this compound is an antioxidant, but has been shown to stabilise the cytosolic GSTs probably by occupying the active site.(Graminski *et al* 1989 and Principito *et al* 1988). However, no increase in activity was observed in these experiments.

Other less aggressive methods are available for removal of the yeast cell wall, such as enzymatic treatments. Zymolase was used to create spheroplasts which were then lysed osmotically with additional sonication or homogenisation (Section 2.4.1). However the yield of material was poor and activity values obtained were lower than with the previous method. Maximum expression was observed in the late log. and stationery phase, at this point the yeast cease to divide and the cell wall thickens, this makes its removal with enzyme inefficient. Therefore it would appear that activity obtained is, to some degree, dependent on the method of preparation, which suggests that some inactivation may have occurred on preparation of the membranes from yeast.

These studies do not demonstrate conclusively whether the recombinant microsomal GST is partially inactive, or was inactivated during isolation of subcellular fractions.

4.4.6. The effect of codon usage on the expression of the microsomal glutathione S-transferases in *S.cerevisiae*.

The choice of the use of a codon for a particular amino acid varies amongst organisms. Poor levels of expression of foreign genes in organisms with markedly different codon usage has been offered as an explanation for low levels of expression. The northern blot analysis in Figure 4.2 showed that the level of RNA in yeast was higher than in rat liver, but subsequent protein expression was found not reflect this

difference. This observation suggests that the rat microsomal GST is not efficiently translated in *S.cerevisiae*.

Of the 61 possible coding triplets there are 25 codons which appear to be used in preferentially in all sequenced *S.cerevisiae* genes (Bennetzen and Hall 1981). The levels of the isoacceptor t-RNA (transfer RNA) species correspond to the preferred codons and are present in excess over the other t-RNA species. The occurrence of the preference codons varies from protein to protein. However the level of expression correlates to the usage of the 25 preferred codons. For example, the highly expressed glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase 1 (ADH 1) genes the preference codon representation is greater than 90%. This falls to 20% in the poorly expressed iso-2-cytochrome c. From these results a rare codon and low abundance of tRNA may be a rate limiting step in protein translation (Bennetzen and Hall 1982).

To investigate the effect of codon usage on recombinant protein expression, the yeast PGK gene was expressed on a multicopy plasmid. Under these conditions the PGK protein was expressed at 20-30% of total cellular protein. Mutation of the triplets encoding the first 25 amino acids to unpreferred codons had no change on the level of expressed protein. However protein expression was essentially abolished when 30% of the protein, the entire 5' end was altered (Hoekeman *et al* 1987). It has also been shown that the first 79 codons of the PGK coding region are required for maximal transcription (Mellor *et al* 1987). So called downstream activation sequences (DAS) have been observed in other proteins (Purvis *et al* 1987). Again there are exceptions to the rule as illustrated by the foreign proteins, hepatitis B core antigen (HBcAg) and β galactosidase, which express highly in yeast, this is probably a function of their intrinsic stability.

Figure 4.8a shows the yeast codon bias in all three frames as applied to the rat microsomal GST. For comparison the codon preference of the highly expressed yeast phosphoglycerate mutase (PGM) gene is shown in figure 4.8b. When the open reading frame (ORF) of PGM is encountered there is a dramatic increase in usage of preferential codons, however no such favourable usage is observed in the microsomal GST

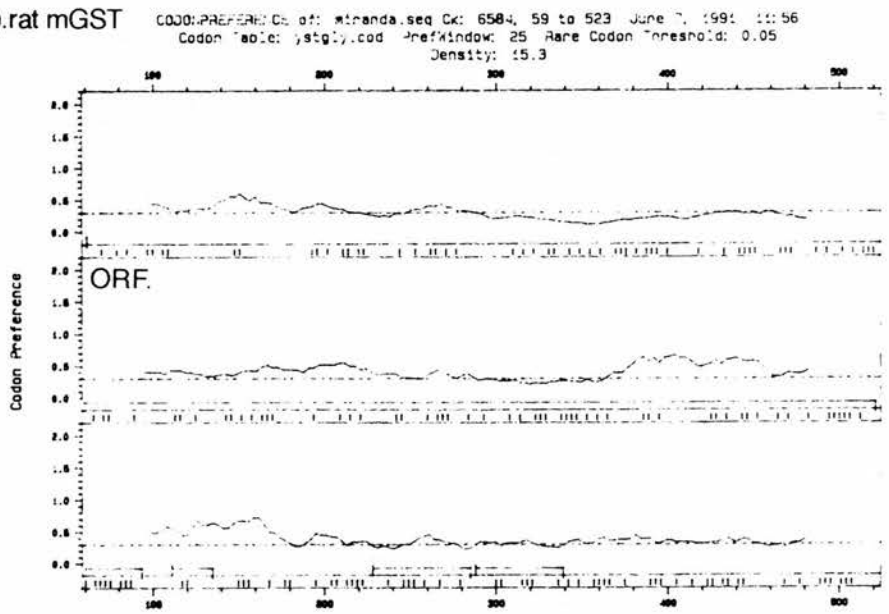
ORF. In fact there is no appreciable difference between coding and non coding regions. The computer programme was based on yeast glycolytic enzymes, so naturally PGM will give high values.

Figure 4.9 shows the correlation between the extent of codon usage bias towards the 25 preferred codons and the level of mRNA in yeast cells for 8 yeast genes (Bennetezen and Hall 1982). Included in the Figure is the yeast codon usage bias calculated for the rat microsomal GST, as can be seen the value of 0.19 falls in the range for genes which are expressed at low levels in yeast cells.

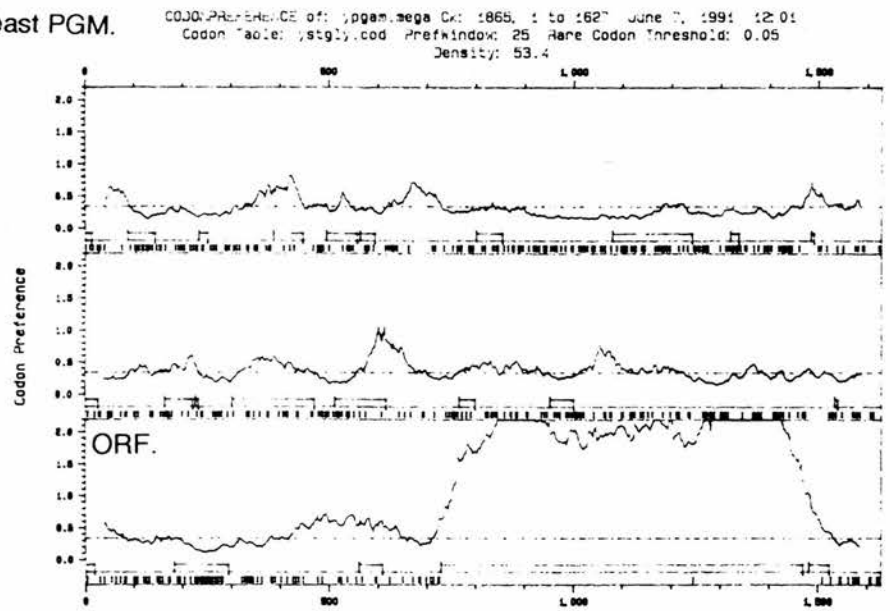
The actual limitations of codon usage on foreign gene expression have not been fully investigated, but it is worth remembering the exceptions cited. However, it can be concluded from this analysis that the microsomal GST does not posses a favourable codon bias in *S.cerevisiae*.

Figure 4.8. Graphical representation of yeast codon bias usage in the rat microsomal glutathione S-transferase and yeast phosphoglycerate mutase.

a).rat mGST



b).yeast PGM.



PGM: phosphoglycerate mutase.

mGST: microsomal GST.

ORF: open reading frame.

Figure 4.9. The codon bias index and the approximate cellular mRNA levels for eight yeast genes.

Gene	Codon bias index.	Approximate % of total cellular mRNA.
pgap 63 (G3PDH)	0.99	1.5-6
pgap 491 (G3PDH)	0.98	
peno 8	0.96	1-3
peno 46	0.93	
ADH-1	0.92	0.7-2
Histone 2B		
(H2B1)	0.75	0.4
(H2B2)	0.68	0.4
Iso-1 cytochrome c	0.50	0.05
rat microsomal GST	0.19	
Iso-2 cytochrome c	0.15	0.003

The codon bias index was calculated as described in Bennetzen and Hall 1982.

4.4.7. Optimisation of the expression of the rat microsomal glutathione S-transferase from the pYEDP10-1 expression construct.

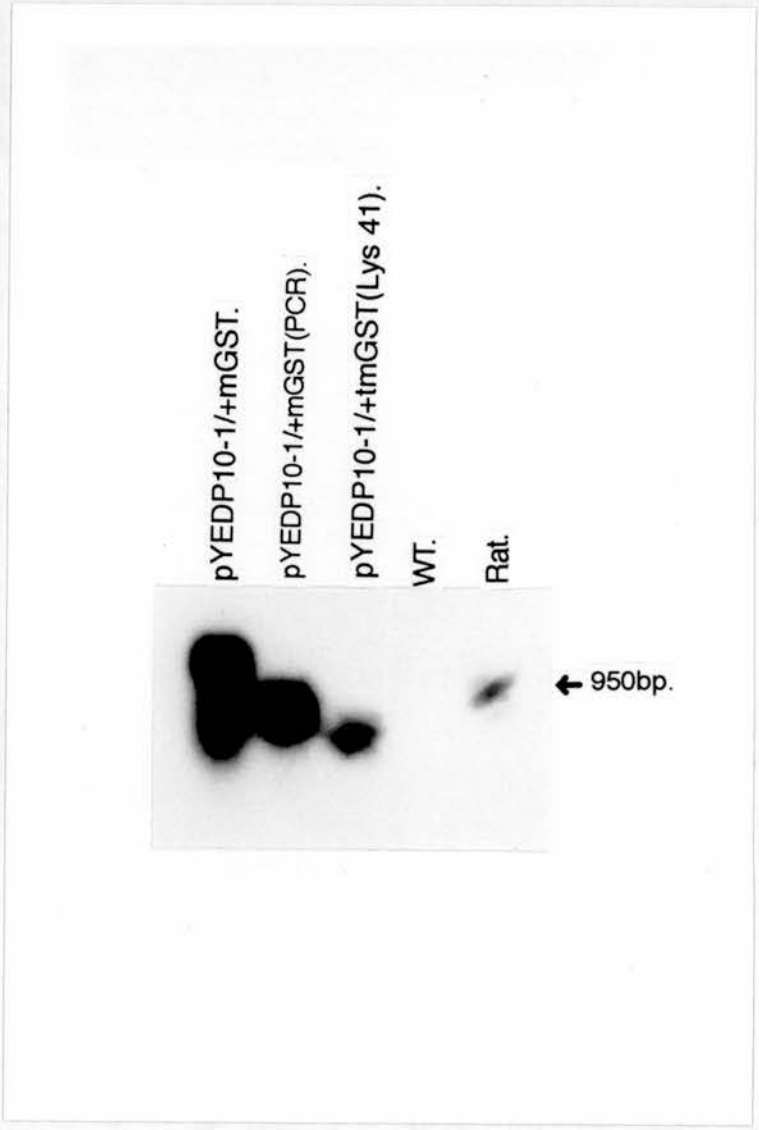
4.4.7a. Removal of rat microsomal glutathione S-transferase 3' and 5' sequences from the pYEDP10-1/mGST expression construct.

In the use of pYEDP10-1 (and pYEDP1/8-2) in the expression of mouse P450 P1 the distance of the ATG from the *Bgl* II site in the vector was shown to influence expression (Cullin and Pompon 1988). When the original vector, pMA91, was constructed a *Bgl* II site was inserted downstream of the PGK promoter at position -2bp by a 6bp linker (Mellor *et al* 1983). The highest levels of mouse P450 P1 expression were achieved when the ATG proximal to the *Bgl* II site. The level of expression was diminished to a third by insertion of 10bp between the ATG and the *Bgl* II (see figure 4.12) The GAL 10 promoter proved more susceptible to these changes (Cullin and Pompon 1988). The authors suggested that the level of translation was increased by shortening the distance between the ATG and *Bgl* II site, However no northern blot analysis was shown to demonstrate that transcription was not affected. As the translation of the microsomal GST message in *S.cerevisiae* appeared to be inefficient it seemed appropriate to remove the 28bp at the 5' end of the cDNA between the *Bgl* II site and the ATG in the cDNA.

In order to reduce the distance between the ATG and *Bgl* II site in pYEDP10-1/+mGST oligomers were designed complementary to the ATG and preceding coding 20bp (incorporating a 5' *Bgl* II site) and the last 20bp and stop codon (incorporating a 3' *Eco*RI site). Using polymerase chain reaction (PCR) it was possible to produce a microsomal GST clone in which all the 5' and 3' sequences had been removed and facilitate directional cloning into pYEDP10-1. Removal of the 3' region of the cDNA would mean the yeast PGK terminator sequences would be operative.

Figure 4.10 shows the Northern blot analysis of the rat microsomal GST mRNA produced from the original construct (pYEDP10-1/+mGST)

Figure 4.10. Northern blot analysis of the transcripts from three differently derived rat microsomal glutathione S-transferase cloned into pYEDP10-1 and expressed in yeast.

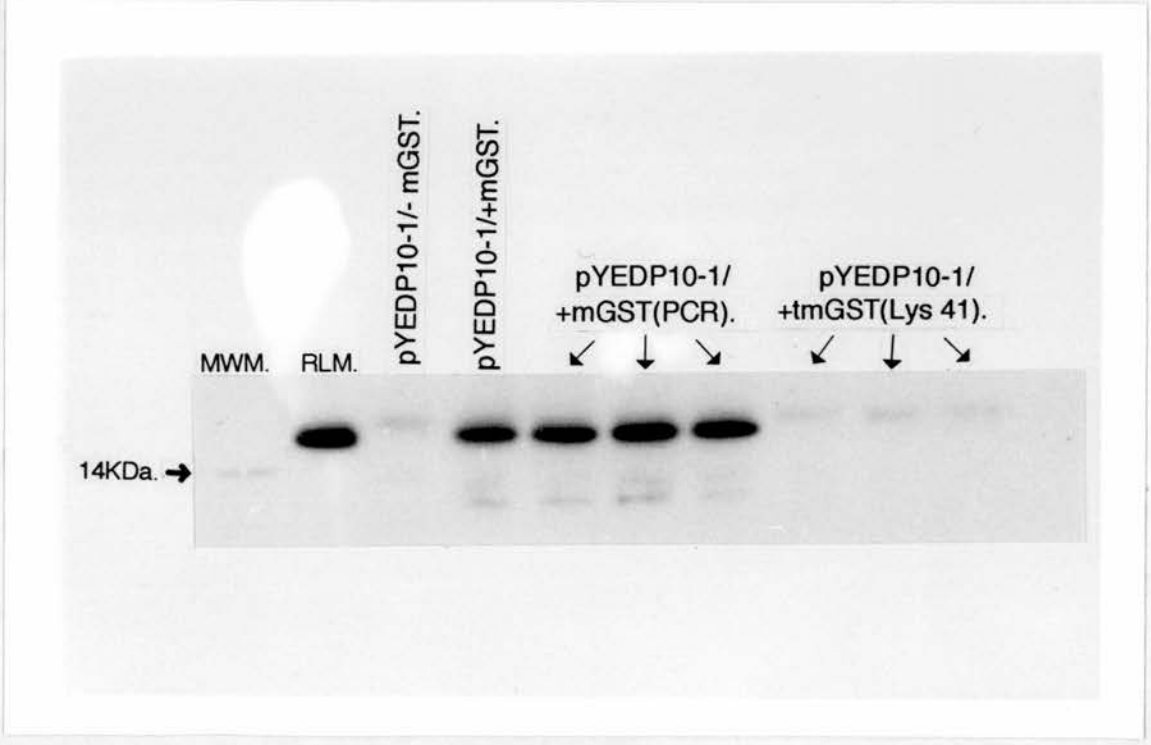


10 μg of total RNA loaded.

WT.: wild type.

Details of the three constructs are discussed in the text.

Figure 4.11. Western blot analysis of the transcripts from three differently derived rat microsomal glutathione S-transferase cloned into pYEDP10-1 and expressed in yeast.



MWM: molecular weight markers

RLM: 5μg of rat liver microsomal protein loaded.

50μg of yeast cell protein extract loaded.

Details of the three constructs are discussed in the text.

and a construct containing PCR cDNA [pYEDP10-1/+mGST(PCR)]. As can be seen no increase in transcription was observed by removing the 5' non-coding region in the cDNA or by removing the 3' non-coding sequences. The RNA from the cDNA without the 3' end of the rat non-coding sequence shows less degradation and the second transcript (at~550bp) is also absent.

Figure 4.11 shows the Western blot analysis of the protein produced from the original construct and three constructs containing PCR cDNAs, as can be seen, there was no change in the level of protein expression reflecting the mRNA observations.

The result of this manipulation did not affect the transcription and /or the translation of the rat microsomal GST in the pYEDP10-1, although previous work had shown the same alterations to increase the level of expression of the mouse P450 P1 (Cullin and Pompon 1988)

4.4.7b The 5' consensus sequences for the initiation of translation in *S.cerevisiae.*

The sequence directly 5' to the initiating AUG has also been studied with reference to translation (Hamilton *et al* 1987). Protein synthesis starts at 5' to the AUG codon. After the 40s ribosomal subunit binds to the Cap site, the subunits scan the leader sequence until the first AUG codon is encountered (Kozak 1984). The importance of the sequence around the AUG codon is illustrated by consensus sequences which are found in mammals (Kozak 1984), plants (Hiedecker and Messing 1986) and yeast (Hamilton *et al* 1987). In this context the two microsomal GST cDNAs and the P450 P1 cDNAs used in pYEDP10-1 were examined.

Figure 4.12 shows the yeast consensus sequence compared with the two 5' microsomal GST sequences and the mouse P450 P1 sequences (from Cullin and Pompon 1988) and for reference the mammalian 5' consensus sequence is included (Kozak 1984). The -3bp A is conserved in all yeast and 81% of mammalian sequences. Of the cDNAs studied this is only found in the wild type microsomal GST. In general mammalian translation initiation sequences are C rich, whereas

Figure 4.12:- 5' Consensus sequenses in *Saccharomyces Cerevisaie* and Mammalian genes.

Position.	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	Relative level	bp from
Occurrance.	67%		52%		100%	50%	89%				38%	52%	57%	of protein	Bgl II site in
[a] Yeast	**A**	**A**	**A**	**A**	A	**A**	A	**A**	**U**	**G**	**U**	**C**	**U**	expression	pYEDP1 10-1
consensus.	G	**U**		**C**		**C**							**C**	within cDNAs	
[b]mGST/WT.	U	G	**A**	**A**	A	G	C	**A**	**U**	**G**	G	**C**	**U**	1	28
mGST/PCR.	**A**	**A**	G	**A**	U	C	U	**A**	**U**	**G**	G	**C**	**U**	1	0
[c]P1(+9bp).	G	C	U	**A**	U	C	U	**A**	**U**	**G**	**U**	A	**U**	4	9
[c]P1(+0bp).	**A**	G	G	**A**	U	C	C	**A**	**U**	**G**	**U**	A	**U**	6	0
[d]P1(WT).	G	C	C	U	U	C	C	**A**	**U**	**G**	**U**	A	**U**	-	-

Occurance.			81%						
[e]Mammalian consensus.	**C**	**C**	**A** / **G**	**C**	**C**	**A**	**U**	**G**	**G**
mGST/WT.	A	A	**A**	G	**C**	**A**	**U**	**G**	**G**
P1(WT).	**C**	U	U	**C**	**C**	**A**	**U**	**G**	U

HIGH BIAS (89-100%)

CONSENSUS

NON CONSENSUS

NEVER FOUND

[a] Hamilton et al NAR **15** 3581-3581 (1987).

[b] mGST=rat microsomal GST Dejong et al JBC 8430-8436 (1988).

[c]Cullin et al Gene **65** 203-217 (1988).

[d] P1=mouse P450 P1 Kimara et al JBC **259**10705-10713 (1984).

[e]Kozak NAR **12** 3873-3893 (1984).

yeast are A rich. An A is found at 40% of positions in this region in yeast, which suggests an unfolded RNA structure is required for translation. As the content A residues in this region is reduced a decrease in the level of expression is observed (Hamilton *et al* 1987). This is dramatically illustrated by the example of the expression of the hepatitis virus coat protein gene in *S.cerevisiae,* the AUG is preceded by 10 G residues, when these were removed protein expression was increased 100 fold (Hamilton *et al* 1987).

On crude analysis of the sequences displayed the wild type microsomal GST 5' sequence contains the -3bp A, which is lost in the PCR product. The lower expressing P450 P1 clone contains a G at position -7bp, which is replaced with a favourable A in the higher expressing construct. There is no data on the effects of such micro-changes on protein level expression, but it is worth considering that neither the distance or the nucleotides leading up to the ATG can be entirely held responsible.

The codon triplet directly after the AUG is thought to influence the stability of both the protein and the efficiency of mRNA translation (Hamilton *et al* 1987). The stabilising amino acids are Met, Ser, Ala, Gly, Thr, and Val encoded by the bias usage of A/U/GCU. The significance of the stability of these codons is explained by the N-terminal rule as mentioned earlier. The second amino acid in the microsomal GST is Ala, which is encoded by GCU, a stabilising codon with a favourable yeast codon bias. Recently the contribution of the ubiquitin degradation pathway to protein turnover in yeast has been questioned (Romanos *et al* 1992).

As an aside it is worth considering the 5' mammalian consensus sequence with respect to the microsomal GST. It would appear that it does not fit particularly well, even at a gross level as it is not C rich (even further in the 5' region). DeJong *et al* 1988 commented on protein expression being low relative to the mRNA levels (as discussed in Chapter 3). There is evidence of additional suppression of translation involving the 5' region of the human microsomal GST. At position -33 to -31bp upstream of the ATG there is an out of frame ATG, when translated this produces a hexapeptide which is terminated at position+20 to+22bp

by a TAA (DeJong *et al* 1988). This arrangement would depress the initiation from the authentic ATG (Kozak 1984). No such observations have been made in the rat, but there is an 8bp repeat, AAGATTGA, at positions -23 to -17bp and at positions -12 to -5bp, however, no significance has been attached to these sequences.

4.4.8. The effect of removal of the membrane spanning domain of the rat microsomal glutathione S-transferase on the expression in *S. cerevisiae.*

The potential toxicity and instability of membrane proteins has been discussed in Section 4.21. Interestingly the polyoma virus transformants lost their plasmids or a spontaneous mutation, arose by which the membrane spanning domain was deleted, in order to overcome toxicity (Belsham *et al* 1986).

The microsomal GST can be cleaved with trypsin to produce an activated form which is thought to have undergone a confirmational change similar to the activation by NEM. Therefore, engineering an construct to express a membrane truncated form of the microsomal GST, may not only reduce toxicity (resulting in low levels of protein expression) due to unfavourable interaction with the yeast membrane, but also produce a more active form of the enzyme.

The putative membrane spanning domain of the rat microsomal GST as judged by the hydrophobicity plot is from amino acid residues 11-35 (Morgenstern *et al* 1985) and Figure 4.17. Limited cleavage with trypsin occurred at portions Lysine 4 and Lysine 41 (Morgenstern *et al* 1989), as Lysine 4 would be found in the lumen of the endoplasmic reticulum, this would indicate that lysine 41 would be the *in vivo* site of proteolysis. There has never been any reports of *in vivo* proteolysis of this enzyme, however oxidative stress was cited as an example of conditions under which this could occur (Chapter 1.4). Activation by proteolysis occurred in both the purified and membrane form, but it was never established whether the membrane "stalk" and cytosolic domain dissociated from one another or not. Membranes exist as a sea of lipids, the bilayer is continually on the move, hence the two peptides may

become separated. On the other hand protein-protein interactions may hold them together. Expression of the cytosolic domain in yeast might increase levels expression and allow investigation of this putative active form of the microsomal GST.

A set of PCR oligomers were designed to produce a truncated form of the microsomal GST protein C terminal of Lysine 41. An ATG replaced Lysine 41 and was immediately preceded by a Bgl II site, the 3' oligo from the Section 4.2.9a was used. This facilitated the direct cloning into pYEDP10-1 and resulting construct was designated pYEDP/+tmGST(Lys41).

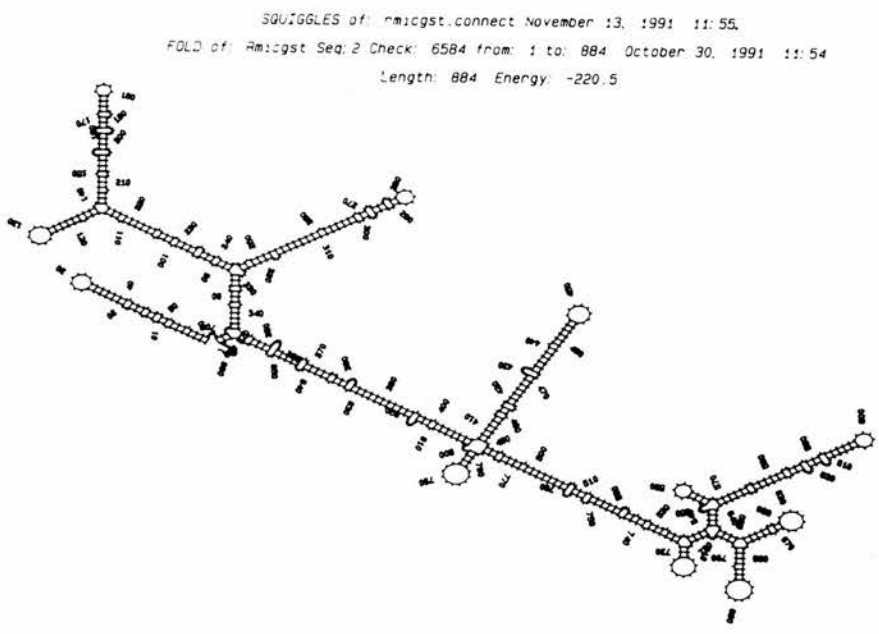
Figure 4.10 shows the Northern blot of the mRNA produced by the PCR generated full length clone minus 5' and 3' sequences and the wild type cDNA. Also, the transcript from the truncated form of the microsomal GST is shown. The level of mRNA observed from the construct expressing the the truncated form of the microsomal GST is greatly reduced. There are two possible explanations for this. Firstly that the efficiency of transcription has been decreased. The sequences 5' of the ATG will be exactly the same the full length microsomal GST clone derived by PCR (section 4.29a), therefore the promoter will be at the same distance from the ATG in both cases. Downstream activating sequences (DAS) in the 5' coding region have been reported to affect the transcription of yeast genes (Mellor *et al* 1987), but these have not been fully characterised or compared with the equivalent regions in mammalian genes.

The second explanation is that the stability of the mRNA has been reduced in the case of the truncated form. A number of factors have been implicated in mRNA stability, such as 5' capping, 3' adenylation protecting against exonuclease attack (Brown 1988), however the afore mentioned clones would share these sequences.

An inverse relationship exists between RNA length and stability. However there are two broad populations of stable and unstable mRNAs, and within these categories the relationship between length and stability exists (Santigo *et al* 1987). The observation here does not agree with this, as the shorter mRNA is diminished. A more recent study into stable and unstable mRNAs showed that they did not significantly differ in

Figure 4.13. The mRNA secondary structure predictions for the full length and membrane truncated rat microsomal glutathione S-transferase.

a) full lenght microsomal GST mRNA.



b). membrane truncated microsomal GST mRNA.

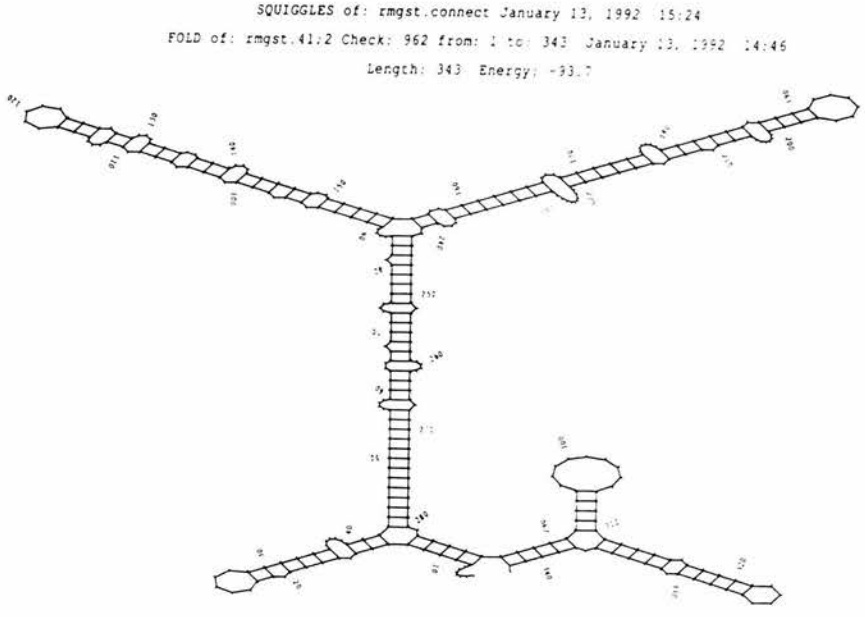
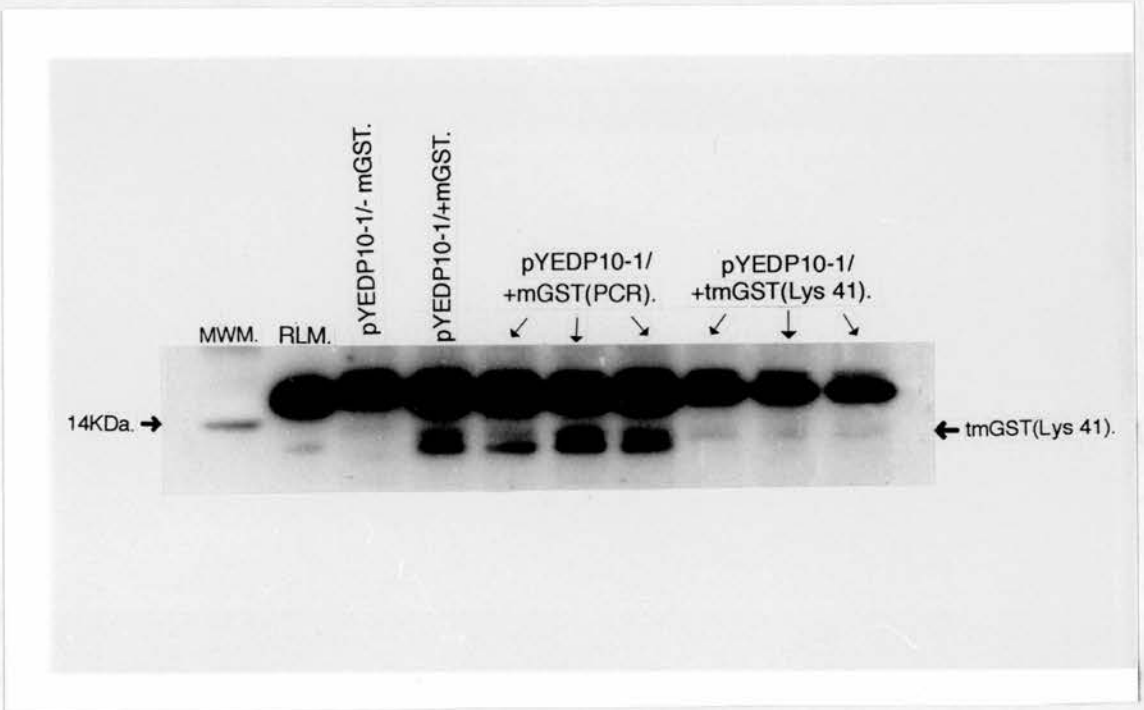


Figure 4.14. The expression of the membrane truncated rat microsomal glutathione S-transferase [tmGST(lys41)] in yeast.



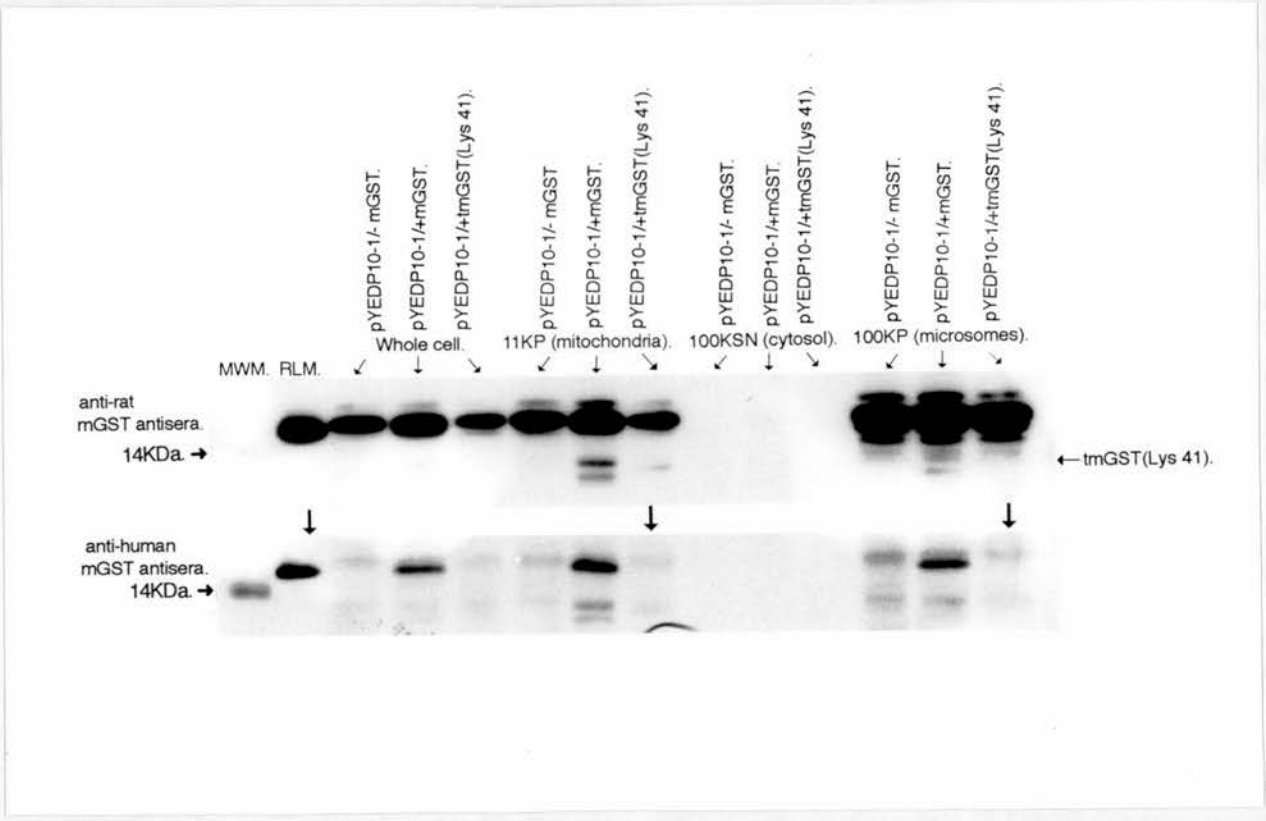
MWM: molecular weight markers

RLM: 5μg of rat liver microsomal protein loaded.

50μg of yeast cell protein extract loaded.

Details of the three constructs are discussed in the text.

Figure 4.15. Subcellular localisation of the membrane truncated and full length rat microsomal glutathione S-transferase expressed in yeast.



MWM: molecular weight markers

RLM: 5μg of rat liver microsomal protein loaded.

50μg of yeast cell protein extract loaded.

Details of the three constructs are discussed in the text.

poly(A) metabolism; nor did adenylation affect stability; nor was their any relationship between size and stability. The degradation of both stable and unstable mRNA depended on concomitant translation elongation, and also the percentage of rare codons was higher in unstable mRNA (Herrick *et al* 1990).

Aberrant proteins in which premature stop codons have been inserted show destabilisation of message. The yeast URA1 and URA3 mRNA were destabilised by the insertion of premature stop codons (Pelsy and Lacroute 1984; Losson and Lacroute 1979). In the presence of a nonsense suppressor the stability of the URA3 message was restored to nearly that of the wild type (Losson and Lacroute 1979). Brown (1989) infers that mRNAs of the translated aberrant proteins are recognised and degraded. Although no premature stop codon has been inserted here, an aberrant protein has been created. This would seem the most analogous observation to the situation presented here.

Figure 4.13a and 4.13b show the secondary mRNA structure predictions for the full length cDNA and for the membrane truncated form (sequences derived from DeJong *et al* 1988). The structures are markedly different, this is hardly surprising due to the removal of sequence in the truncated form. However the wild type sequence appears to be intrinsically more stable (energy value -220.5kcal/mol) as compared with a value of only -93.7kcal/mol for the truncated transcript. However this is purely a computer prediction based on obtaining the energy minimum, and it does not take into cellular factors such as RNA binding proteins. It may be reasonable to assume that gross changes in secondary structure and an energetically less favourable conformation could also cause instability of the message.

Figure 4.14 shows the Western blot analysis of protein prepared from yeast expressing the truncated microsomal GST and the low level of the protein is a reflection of the low levels of the mRNA. This blot is in fact loaded with identical samples as Figure 4.11, but it is probed with the anti-rat microsomal GST anti body as supposed to the anti-human antisera. For the truncated form to be seen a long exposure is required and the cross-reacting yeast band is seen to comigrate with the expressed microsomal GST band (as discussed in section 2.75). The

predicted molecular weight of the truncated microsomal GST, from the amino acid sequence, is 12.67KDa. Although the band is faint it is not observed in the control yeast. The yeast expressing the full length sequence show two major proteolytic products in this region, indicating a protease sensitive site. Other proteases have been shown to cause proteolysis in this region (Morgenstern *et al* 1989, Lundqvist and Morgenstern 1992). From the hydrophobicity (Figure 4.17) this is the only major hydrophilic region of the protein. Interestingly a band can also be seen in this region in the rat liver microsome sample. It cannot be ascertained whether this is non specific cross-reactivity (often associated with long exposure of autorads), or in *vivo* proteolysis or degradation that occurred during microsome preparation.

Figure 4.15a shows subcellular fractions from yeast transformed with pYEDP10-1/-mGST, pYEDP10-1/+mGST and pYEDP10-1/+tmGST(Lys41) probed with the anti-rat microsomal GST antisera. The truncated microsomal GST was located in the 11000g pellet, however it was not possible to determine its presence in the other membrane fraction due to non-specific cross-reactivity observed with this antibody. The same samples probed with the anti-human microsomal GST anti sera is include as a reference (Figure 4.15b). The truncated species was never found in the cytosolic fraction, and this is in keeping with Morgenstern's observation on treating rat liver microsomes with trypsin. (Morgenstern *et al* 1989).

4.4.9. Fusion of the truncated rat microsomal glutathione S-transferase with portions of *S. cerevisiae* alcohol dehydrogenase 1.

In the above studies a number of factors affecting the stability of both protein and RNA have been considered. The N terminal region of the protein has been cited in relation to degradation by the ubiquitin-pathway. Cytosolic proteins tend to be more stable having small uncharged amino acids (Ser,Ala,Gly, and Val encoded by (A/U/GCG). The rat microsomal GST contains an alanine encoded by GCU at the N terminus, but the truncated form contained an N terminal valine encoded

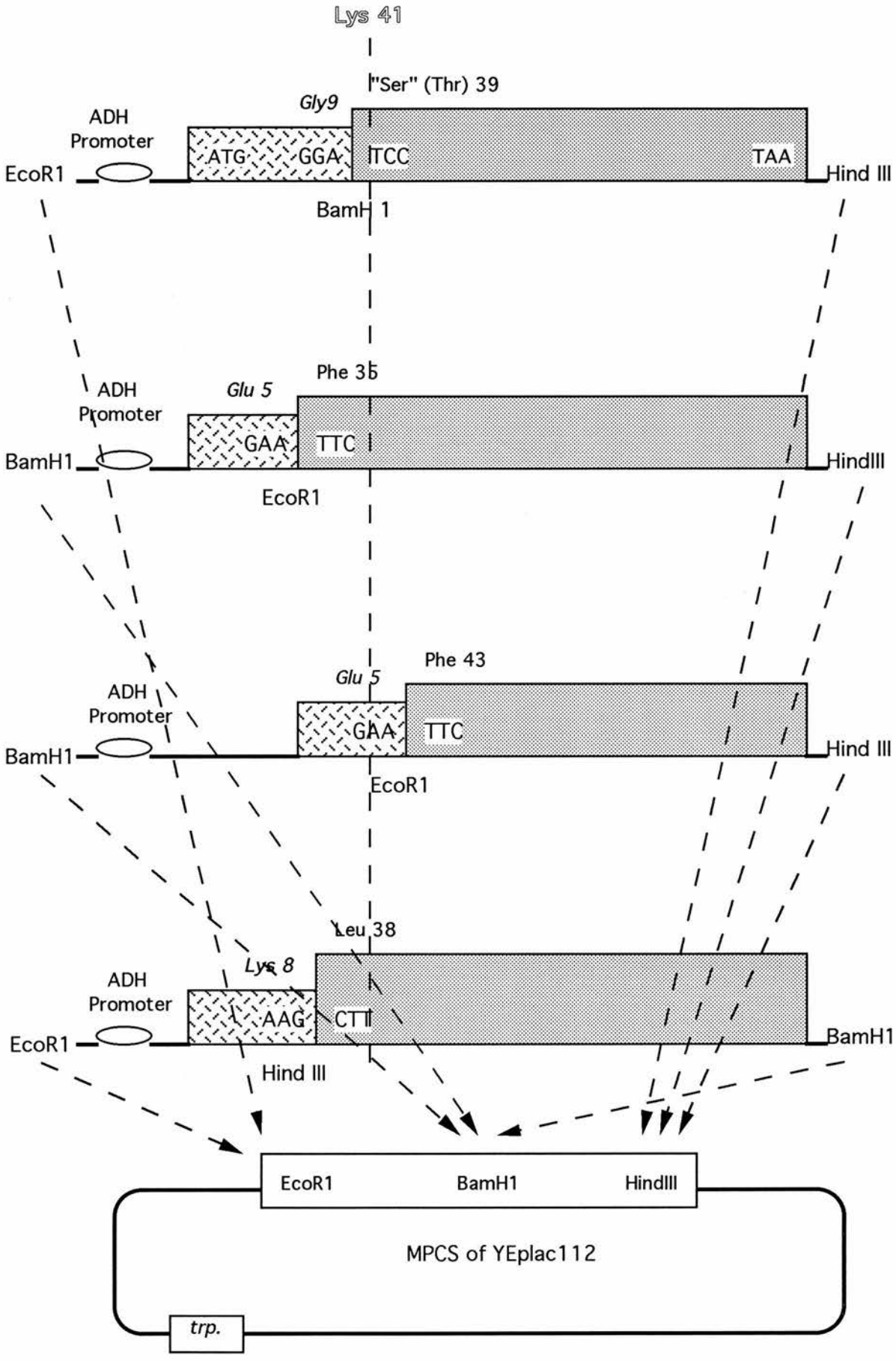
by GUU, which is an unfavourable codon bias. The codon for the first amino acid is thought to affect the efficiency of translation of the the protein (Hamilton *et al* 1987).

Fusion of an unstable protein to a more stable one has been reported to allow successful accumulation of the hybrid, as exemplified by fusions with the previously mentioned superoxide dismutase and hepatitis B core antigen (Cousens *et al* 1987, Beesly *et al* 1990). The stability of the rat P450 reductase expressed in yeast was increased by swapping the rat membrane spanning domain with the sequence encoding the yeast endogenous P450 reductase (Bligh *et al* 1992), however this may have had an effect by enhancing insertion into the membrane.

The lysine 41 truncated microsomal GST was in effect a "cytosolic" protein so fusion to a cytosolic protein would seem appropriate. A small amount of yeast protein might allow a more favourable initiation of translation as well as increase stability of the protein. The entire alcohol dehydrogenase 1 gene (ADH) was available in the plasmid pJD14 (gift from A.Boyd). This meant a construct could be created using the ADH promoter sequences and all the 5' untranslated sequences. The fragment of microsomal GST was only 12KDa long, so the amount of ADH protein used was kept to a minimum, as a large fragment might compromise the folding of the microsomal GST. A range of fusions were created containing between the first 4 and 8 ADH amino acids.The fusion with the microsomal GST was made in the region of the lysine 41 residue, because suitable a codons were required to form restriction enzyme sites. The "hybrid" cDNA containing the ADH I promoter was cloned into the multiple cloning site of YEplac 112, a 4.99kb 2μ based episomal plasmid (Gietz and Sugino 1988). Figure 4.16 shows the cloning strategy for the above construction of these fusion products. Figure 4.17 shows the hydrophobicity plot of the microsomal GST, as can be seen, the fusion with the ADH N terminal amino acids were made in the hydrophilic region of the protein. A fusion with the full length microsomal cDNA and the first four amino acids of ADH was also made.

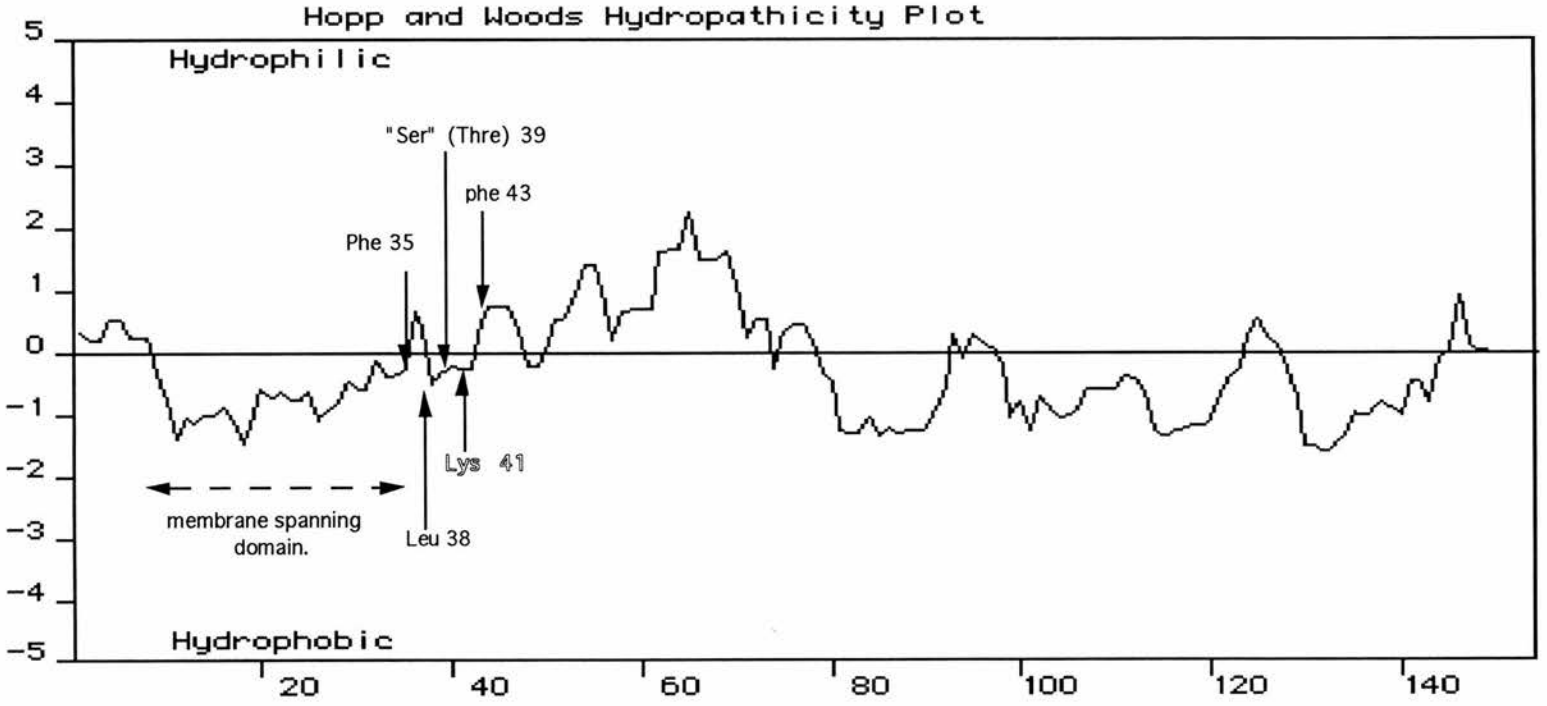
Figure 4.18 shows the Northern blot analysis of the fusion protein constructs probed with the rat microsomal GST cDNA. There appears to

Figure 4.16 Construction of ADH and microsomal glutathione S-transferases fusion proteins.



The digram is not drawn to scale

Figure 4.17 Hydropathicity plot and amino acid sequence of the microsomal GST and ADH fusion proteins. pg146



10 20 30 40 50 60 70

mGST*WT* ☞ ADLKQLMDNE VLMAFTSYAT IILAKMMFLS SATAFQRLTN KVFANPEDCA GFGKGENAKK FLRTDEKVER ☞

SIPETQKG SN KVFANPEDCA ⇔ *Gly9* / Ser 39

SIPE FQRLTN KVFANPEDCA ⇔ *Glu5* / Phe 35

S I PE FANPEDCA ⇔ *Glu5/* Phe 43

SIPETQK LTN KVFANPEDCA ⇔ *Lys 8* / Leu 38

*S IPEFRM*ADLKQLMDNE ⇔ *Glu5/* mGST full length.

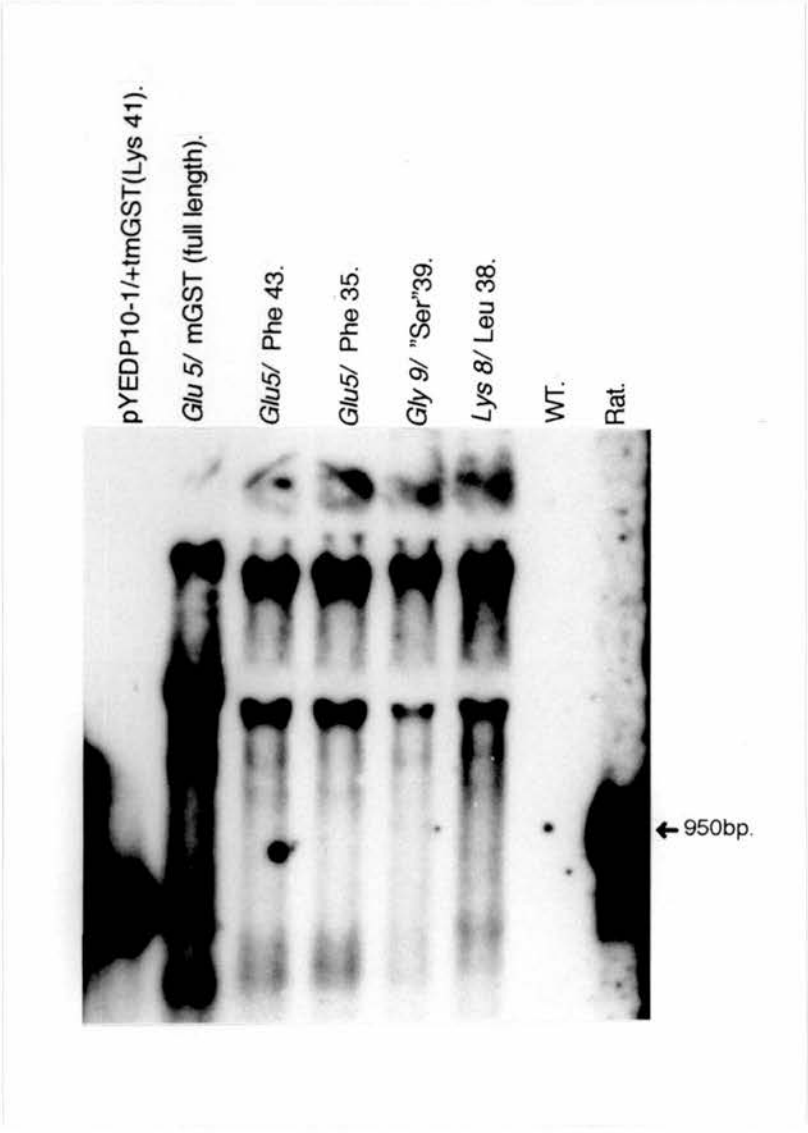
80 90 100 110 120 130 140 150

☞ VRRAHLNDLE NIVPFLGIGL LYSLSGPDLS TALIHFRIFV GARIYHTIAY LPLPQPNRG LAFFVGYGVT LSMAYRLLRS ☞

☞ RLYL.

BASIC residues HRK HYDROPHOBIC residues ALIV ACIDIC residues DE *ADH sequences* MICROSOMAL GST sequences

Figure 4.18. Northern blot analysis of yeast transformed with ADH/microsomal glutathione S-transferase fusion constructs.



10μg of total RNA loaded.

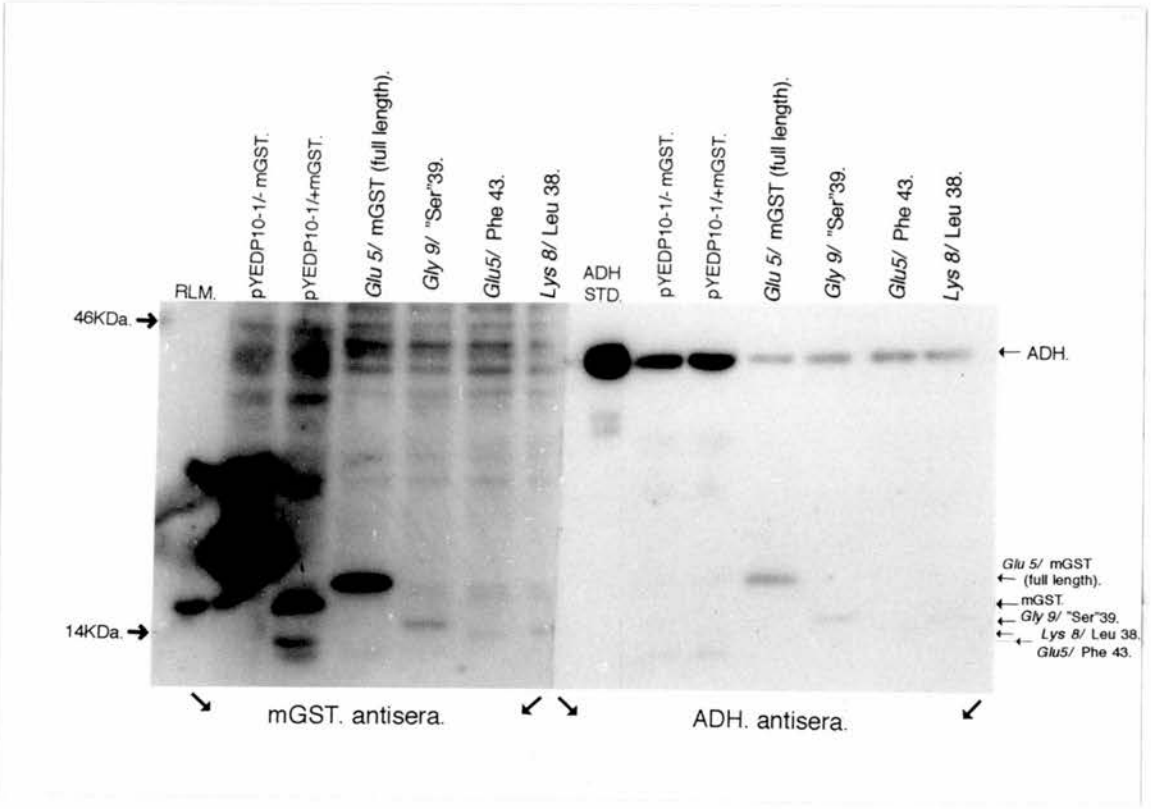
Details of the three constructs are discussed in the text.

be two transcripts present from the constructs containing the fusion proteins. The ^{32}P signal is blocked at in two regions, which is probably due to large amounts of ribosomal RNA transferred in these regions, which would compete with the mRNA from the fusion proteins. The existence of two transcripts can not be explained, nor is it known if protein is translated from both, also it is not possible to determine whether increased transcription was achieved in this system. As a point of interest there were no 3' non-coding regions from the microsomal GST or ADH cDNAs (or any other terminator) incorporated into these constructs, but two distinct transcripts still exist.

Figure 4.19 shows a western blot loaded in duplicate. Half is probed with the anti-human microsomal GST the equivalent samples in the other were probed with the anti-ADH I antibody. This demonstrated that fusion protein constructs expressed sequences from both parent proteins. Figure 4.20 shows a western blot whole cell extracts from the strain expressing the fusion proteins; the truncated version and the full length cDNA under the PGK promoter. The controls are as before except yeast transformed with the parent plasmid YEplac 112 for the fusion proteins is included. No increased expression over the truncated form had been achieved by the creation of the fusion proteins. The anti-rat microsomal GST antibody was used to probe this blot, again the yeast cross reacting band and the expressed microsomal GST band have not been fully resolved.

[Analysis of subcellular localisation was carried out for the fusion proteins and the Western blot was probed with the rat microsomal GST antibody. Again the proteins appeared to be in the membrane fractions and not the cytosol. Too many cross-reacting bands were present in the 100 000g pellet to establish the presence of these protein species, however they were definitely present in the 11000g pellet. The results are shown, for one fusion protein *Glu5*/phe35, in Figure 4.21. The proteolytic fragment produced by the action of trypsin on the native rat microsomal GST also remains associated with the membrane (Morgenstern 1989). The hydrophobicity plot illustrates the remainder of the protein is highly hydrophobic, this property may causes them to interact with the membranes. Although the remainder of the protein is

Figure 4.19. Western blot analysis of yeast transformed with ADH/rat microsomal glutathione S-transferase fusion constructs using human microsomal glutathione S-transferase and yeast alcohol dehydrogenase antisera.



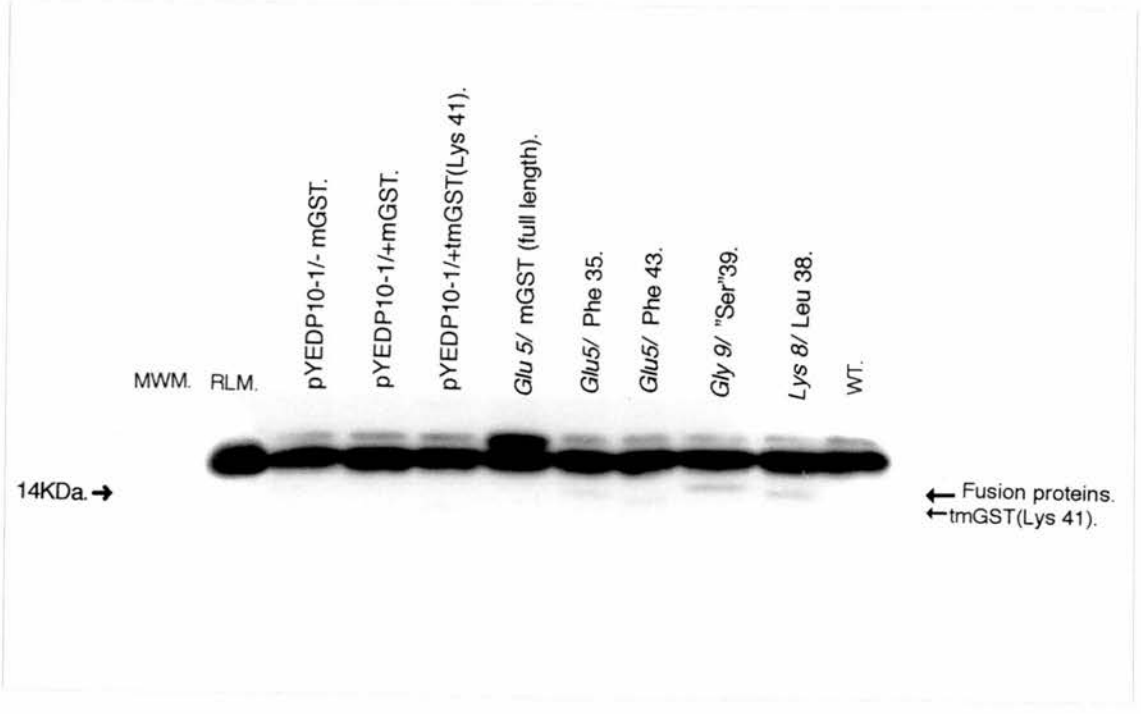
MWM: molecular weight markers

ADH STD: ADH standard- 1μg of purified yeast ADH (Sigma) protein loaded.

50μg of yeast whole cell protein extract loaded.

Details of the three constructs are discussed in the text and shown in Figure 4.16..

Figure 4.20. Expression of the ADH/microsomal glutathione S-transferase fusion proteins, full length and membrane truncated microsomal glutathione S-transferase in yeast.



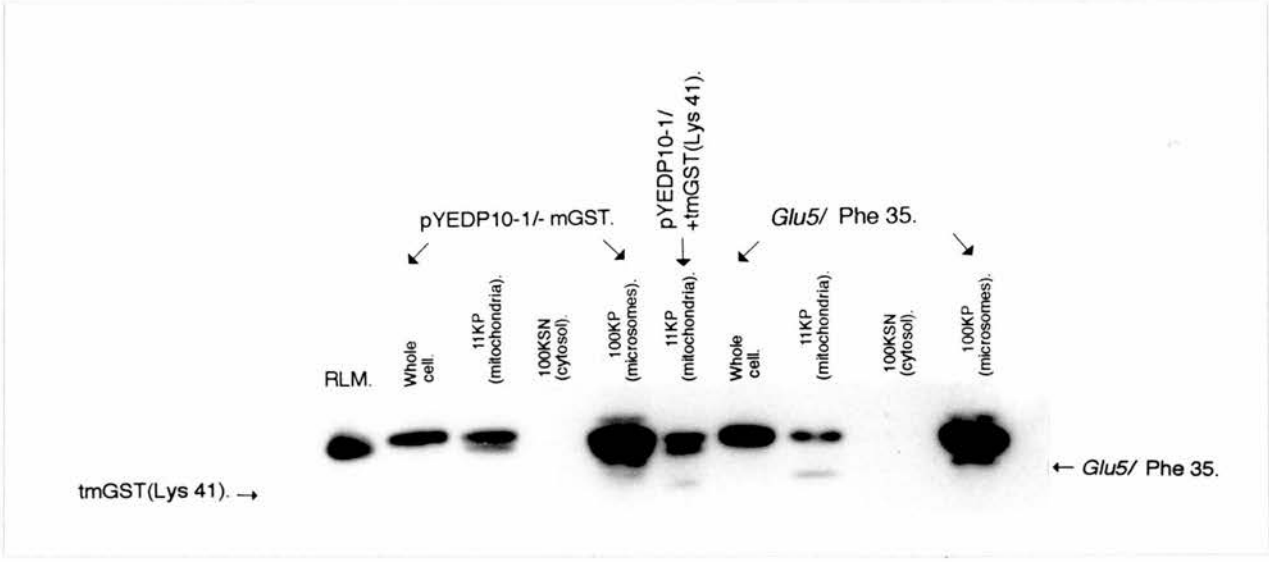
MWM: molecular weight markers

RLM: 5μg of rat liver microsomal protein loaded.

50μg of yeast whole cell protein extract loaded.

Details of the three constructs are discussed in the text and shown in Figure 4.16.

Figure 4.21. Subcellular localisation of the ADH/microsomal glutathione S-transferase fusion protein, *Glu5/*Phe 35 in yeast.



RLM: 5μg of rat liver microsomal protein loaded.
50μg of subcellular fraction protein extract loaded.

hydrophobic there are quite a few acidic and basic residues inter-dispersed in this region. Based purely on this crude examination of the sequence, such charged residues would limit the likelihood of any of these sequences acting as a membrane spanning domain.

From the experiments carried out here it is impossible to draw such conclusions about the nature and cause of the change in subcellular distribution. Firstly it would be necessary to establish whether these were integral or peripheral membrane proteins. To do these studies with a chaotropic agent such as 1M sodium carbonate, pH 11 to remove of peripheral proteins; and treatment with a detergent such as triton-X 100 for the solubilisation of integral proteins, would clarify the nature of these proteins. In addition, in view of the lack of purity of the subcellular fractions was noted in section 4.4.2, a more sophisticated preparation of subcellular organelles would have to be used. The method described by Riezman involving a high resolution separation of orgenelles through sucrose density gradients would be suitable (Riezman *et al* 1983). The antisera used here showed some degree of non-specific cross-reactivity with the wild type yeast. This hindered the identification of the presence fragments in some organelles. Immunopurification, as described in Section 2.7.5, would overcome this obstacle.

The aim of these experiments was to increase expression, this essentially failed. To pursue such studies were not of immediate relevance to the project. However it is an interesting observation that such products are associated with the membrane, which probably reflects the highly hydrophobic nature of the protein.

4.5. Summary.

This section clearly establishes the expression of the rat microsomal GST in *S.cerevisiae* . In accordance with the literature observations the enzyme is located in the membrane fractions. However, analysis of the purity of the membrane fractions demonstrated some cross-contamination between the mitochondria and microsomal fractions and therefore, it is not clear exactly where the protein is expressed.

The expression of the protein was found to be maximal in the latter region of the growth phase. At this point levels were 10-50% of native rat liver expression. The activity however, was lower than the observed protein levels would have predicted. What is not clear is whether the expressed protein was inactivated on preparation of the subcellular fractions or was partially inactive *in vivo.* Purification of the recombinant protein would allow a more accurate determination of specific activity. This determination of activity would be a more informative measure as the yeast membrane preparations are not strictly comparable with rat microsomes as demonstrated by the subcellular marker studies.This process would also remove endogenous inhibitors if present.

The only factor to have any influence on the expression of the microsomal GST in *S.cerevisiae* was the vector, and this was related to the composition of the individual vector rather than the promoter itself. The codon usage of the rat microsomal GST was, as expected for a mammalian protein, found not to have a favourable yeast bias. Removal of 5' and 3' rat non-coding sequences had no effect on expression, nor did altering the first 7bp upstream of the ATG involved in the initiation of translation, alter the levels of mRNA or protein.

The rationale for removing the membrane spanning domain was twofold, firstly to increase expression and secondly to create a putative active form of the enzyme. Unfortunately this drastically reduced the level of transcript and fusion to the yeast cytosolic protein ADH failed to raise them. It was interesting that all the truncated forms remained associated with the membrane, which is probably due to the hydrophobic nature of this enzyme. The exact nature of this association was however not determined.

Chapter 5. The role of the rat microsomal glutathione S-transferase in the metabolism of foreign compounds: using the model *Saccharomyces cerevisiae* expression system.

5.1 Introduction.

Metabolism of a compound can often be complex and involve many steps. Expression of foreign proteins in yeast has allowed the toxicological study of a number of drug metabolising enzymes (as reviewed in section 4.40). There has been much debate in the literature as to the relevance of recombinant expression systems for the study of drug metabolism, because of their inability to activate chemical mutagens. Such claims have prompted the investigation of yeast xenobiotic metabolism. Not surprisingly similar mechanisms have been found in the yeast system as the mammalian system, justifying such experiments.

Although the role of glutathione and its metabolism have been studied in yeast, the details are not as well defined as in the mammalian system. GSH comprises of about 1% of the dry weight of *S.cerevisiae* and plays a leading role in the detoxification of endogeneous oxoaldehydes (Penninkx *et al* 1983) and regulation mechanisms of drug bioreduction (Elkens *et al* 1988). The concentration of GSH and, importantly, the GSH:GSSG ratio is the same as found in mammalian cells (Elskens *et al* 1991; Kosower and Kosower 1978, respectively). Glutathione deficient strains have allowed the study of GSH and sulphur metabolism in yeast and the identification of the enzymes in these pathways. These studies suggested that GSH also acts as an endogenous sulphur source in yeast, as has been proposed for plants (Rennenberg 1982) and animals (Kosower and Kosower 1978; Reed *et al* 1983). The existence of enzymes in these pathways are conserved (Elskens *et al* 1991).

The glutathione-dependent enzymes thought to be involved in xenobiotic metabolism have also been identified in yeast. For example glutathione peroxidase activity has been reported (Casalone *et al* 1988). There are two major forms of peroxidase activity found in mammalian

cells, the selenium dependent and selenium-independent forms corresponding to the glutathione peroxidase level and α class GST respectively. Both of these two peroxidase activities exist in yeast, and in the absence of oxygen the hydrogen peroxidase activity is suppressed, while the organic peroxidase activity is increased. Copper was found to induce both activities (Galiazzo *et al* 1987). Interestingly, strains with low levels showed higher levels of the other activity (Casalone *et al* 1988). A similar observation is made between mammalian species (Ketterer *et al* 1988).

The soluble yeast glutathione S-transferases have been purified from the yeast *Issatchenkia orientalis* (Tamaki *et al* 1989), cloned and sequenced (Tamaki *et al* 1990). The proteins are highly unstable. N-terminal amino acid sequence data reveals the conserved tyrosine 7, essential for catalytic activity (Stenberg *et al* 1991) to be present.

In the previous section the control microsomes were shown to have CDNB activity. To date there has been one highly dubious report of a yeast microsomal GST, where the putative homologue was reported to be a 14KDa associated with porin structure in the outer mitochondrial membrane (Krause *et al* 1986), and also was reported to have GST activity. The antisera to this protein was raised against the 14KDa outer mitochondrial membrane (OMM) protein (Riezmann *et al* 1983), which is solely located in the mitochondria. The purified 14KDa OMM protein was never shown to have CDNB activity (personal communication with Dr.D.Brdiczka:-corresponding author of Krause *et al* 1986). It is of course possible that the endogenous activity observed in the membrane fractions could be due to a yeast form of this enzyme. This would be extremely interesting to study, with respect to the role of the microsomal GST, as cytosolic GSTs appear to be conserved along with their function. The isolation of a yeast homologue to the microsomal GST would enable deletion experiments in yeast to be preformed, which would provide valuable information about the function of the enzyme.

The phase I P450 enzymes have been reported in yeast, but appear to be largely involved in steroid metabolism (Kapelli 1986). However the P450 (phenobarbital-inducible activity) and P488 (3-methyl cholanthrene-inducible activity) activities have been demonstrated in

yeast (Kelly *et al* 1983,1985 and Azari and Wiseman 1982). The P450 and P448 activities appear to be under metabolic control, being increased at high glucose concentrations and in the latter stages of the growth phase (Kelly *et al* 1983). Increased sensitivity to a number mutagens which are known to require P450 and P448 type activation was observed under these conditions, and the addition of Arolclor-induced S9 mix from rats did not elevate sensitivity (Kelly *et al* 1983).

Aflatoxin B_1 (AFB_1) toxicity is mediated by P450 activation. Increased mutagenicity was found during latter phases of growth, again this correlated with changes in endogeneous P450 levels. Further evidence of P450 involvement came from typical inhibition studies. Interestingly, the increase in mutagenicity was increased on raising the incubation temperature from 28°C to 37°C (Naggli *et al* 1986). Two hypothesis were proposed to explain this result. Firstly, uptake of AFB_1 maybe facilitated at higher temperature and secondly the thermolabile nature of the DNA repair process might come into play (Siede *et al* 1984).

5.2 Aims of the chapter.

Chapter 4 described the expression of the rat microsomal GST in *S.cerevisiae*. This chapter covers the use of this system to study a range of putative substrates for the microsomal GST. Various compounds have been tested and the mechanism of action of each is discussed individually.

5.3. Results and Discussion.

5.3.1. Hexachloro-1,3-butadiene.

Amongst the substrates for the microsomal GST hexachloro-1,3-butadiene (HCBD) is probably one of the best characterised. In human liver the glutathione-dependent metabolism is mainly carried out by the microsomal GST (Wallin *et al* 1988, Oesch and Wolf 1989) and the P450 system is not involved (Wallin *et all* 1988). The glutathione conjugation is the initial step in the fate of this compound, further metabolism via the mercapturic acid pathway leads to the production of toxic metabolites. The details of this process were discussed in Section 1.6 (Figure 1.5).

Figure 5.1a and b show two cytotoxicity experiments carried out at 30°C and 37°C, respectively. Other experiments are shown in appendix A. The differences are small, but consistently show an increased sensitivity of yeast expressing the microsomal GST to HCBD. The degree of cell kill observed varies from experiment to experiment, but the incubation temperature had no effect. As discussed in the previous section, the expression of yeast endogeneous drug metabolising capacity can vary according to a number of factors. Many other properties of the yeast cell may alter drug sensitivity, such as changes in cell wall thickness. The variation of expression of the microsomal GST during the growth phase is illustrated in Section 4.4.3. Yeast were subjected to the test compounds drug late log., early stationary phase of growth (OD_{600nm}~1.00). However. variation in protein levels still existed between experiments as shown in appendix B. There are in any *in vivo* system an imponderable number of variables.

In section 4.4.5. the CDNB activity measured did not correlate with the amount of microsomal GST protein present, but it could not be determined whether the protein was fully active in the intact cell or inactivated during preparation. The results here suggest the expressed protein is capable of functioning as an enzyme *in vivo,* although no judgment can be made as to whether the enzyme is entirely active.

It is not the glutathione conjugate of HCBD which is responsible for the ultimate toxicity of this compound, but is due to the metabolite

Figure 5.1a HCBD cytotoxicity experiment conducted at 30oC.

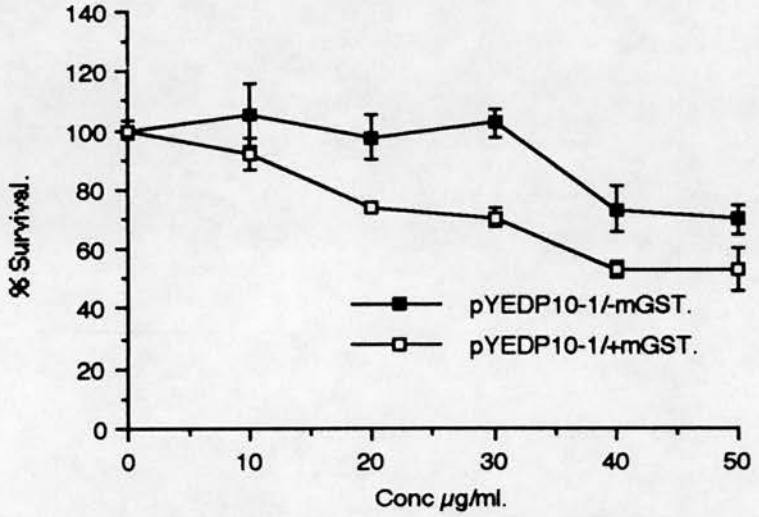
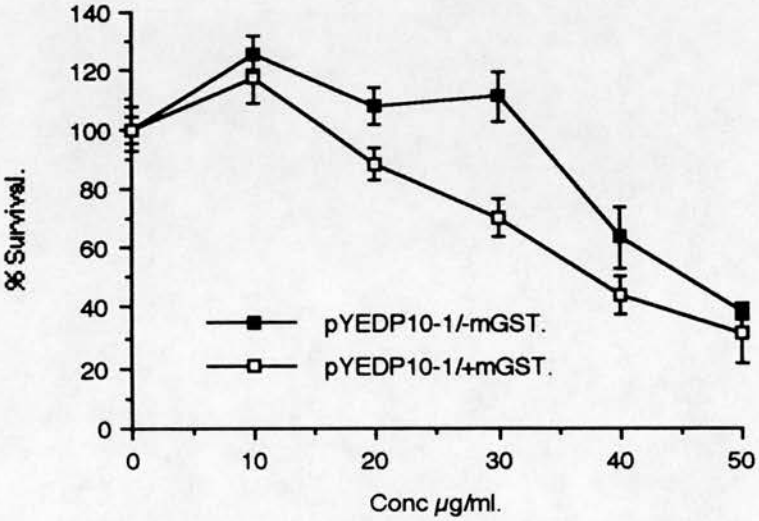


Figure 5.1b HCBD cytotoxicity experiment conducted at 37oC.



The data shown in the above graphs are from single experiments. For each concentration the cultures were plated out in triplicate and from these results the standard deviation was calculated. The error bars shown in the graph are the standard deviations expressed as a percentage of the control value.

formed by the action of a series of enzymes including β-lyase. The results of the present study suggest the required metabolic pathway for toxicity is active in yeast. In mammalian cells toxicity is dependent on the uptake of the glutathione S-conjugates across the brush boarder membrane in the proximal tubule. It is here that the GSH S-conjugates are cleaved by γGT and enter the mercapturic acid pathway. In yeast γGT is a vacuolar enzyme involved in sulphur metabolism (Jaspers and Penninckx 1984). In fact 50% of GSH is located in the vacuole; this is consistent with GSH serving as a storage compound. Substantial portions of amino acids are compartmentalised within the cell, mainly in the vacuole, redistribution of amino acids between compartments in response to metabolic signals has been demonstrated (Wiemken 1980; Messenugy *et al* 1980; Kitamoto *et al* 1980).

In the study of HCBD toxicity in the mammalian cell, modification of the glutathione conjugate by cysteinyl glycine dipeptidase occurs; this enzyme has also been reported in yeast (Jaspers *et al* 1985). An attempt was made to measure β lyase activity in yeast, but this failed due to a high background of endogenous pyruvate and ammonia production. β lyase has been reported in a number of mammalian species and bacteria (Larsen and Stevens 1985), which is not unexpected due to the conserved nature of amino acid metabolism.

5.3.2. 1,2 Dibromoethane.

In chapter 1.2 the ability of certain compounds, or their metabolites, to raise the activity of the microsomal GST was discussed. The microsomal GST activity has been shown to be increased in rats treated with 1,2 dibromoethane (DBE). The activity of microsomal GST in hepatocytes cultivated *in vitro* could also be elevated following exposure to DBE (Lundqvist and Morgenstern 1992b). In contrast to hexachloro-1,3-butadiene this compound can be metabolised by the P450 system as well as undergoing glutathione conjugation. In fact, the majority of metabolite is thought to be produced by the P450 pathway (van Bladeren *et al* 1981) and it is the P450 products which bind to protein (Guengerich

et al 1980). The glutathione conjugate is highly unstable and rearranges to form the episulphonium ion, which is a particularly strong alkylating agent and can bind to DNA (van Bladeren *et al* 1980). All these species are highly reactive and it is hard to imagine that such defined distinction exists and indeed, some interaction between the two pathways has been demonstrated (Guengerich *et al* 1980)

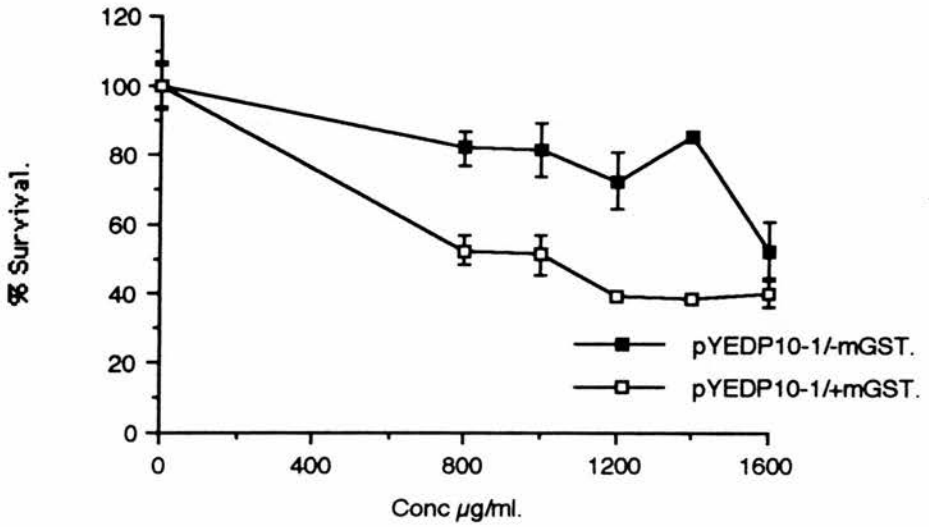
Figure 5.2 shows the cytotoxicity data from a typical experiment. A small increase in sensitivity was observed in yeast expressing the microsomal GST, other examples are shown in appendix C.

The individual capacity of each isoenzyme to conjugate DBE with GSH was assessed (Carmik *et al* 1991), with the rat 2-2, human B_1B_1 and B_1B_2 having activities of 117, 141 and 108 nmol/min/mg respectively. The π and μ and microsomal enzymes having activities of 3, 4 and 1.5 nmol/min/mg. However no purified microsomal enzyme was available and this extrapolated result was felt to be artificially low. Also no measurements were made with activated microsomal enzyme.

The expression of human GST B_1B_1 and π in *Salmonella typhimurium* TA1538 have been shown to cause an increase in mutagenicity (Simula *et al* 1993). Similarly the expression of these enzymes in *S.cerevisiae* causes an increase in cytotoxicity (Black 1989). It appears that the *in vitro* activities do not reflect the *in vivo* observations made in yeast and bacteria. Maybe only a small amount of activity is required for toxicity to manifest itself. High levels of expression were achieved in both these systems, but in the bacterial system the difference in mutagenicity between the two GST isoenzymes did not reflect the difference in enzymic capacities, even when relative levels of protein were considered (Simula *et al* 1993).

In an earlier study using the closely related compound 1,2 dichloroethane (DCE) the relationship between P450 and GST metabolism was investigated (Guengerich *et al* 1980). The NADPH-dependent binding of metabolites to DNA in the microsomes was increased, in a synergistic manner (4-12 times) by the addition of GSH. The DNA binding of metabolites in the microsomes was relatively small in the presence of GSH alone, prior metabolism by P450 brought the

Figure 5.2 DBE cytotoxicity experiment



The data shown in the above graph is from single experiment. For each concentration the cultures were plated out in triplicate and from these results the standard deviation was calculated. The error bars shown in the graph are the standard deviations expressed as a percentage of the control value.

value within the same order of magnitude as the cytosolic GSH-dependent value.

To determine whether the GSH conjugation was occurring enzymatically or non enzymatically, P450 and P450 reductase were reconstituted into liposomes. In these experiments no increase in binding to DNA was observed on addition of GSH. In the microsomal system the increase in binding could not be brought about by either L-cystiene or dithioerythritol (DTT).

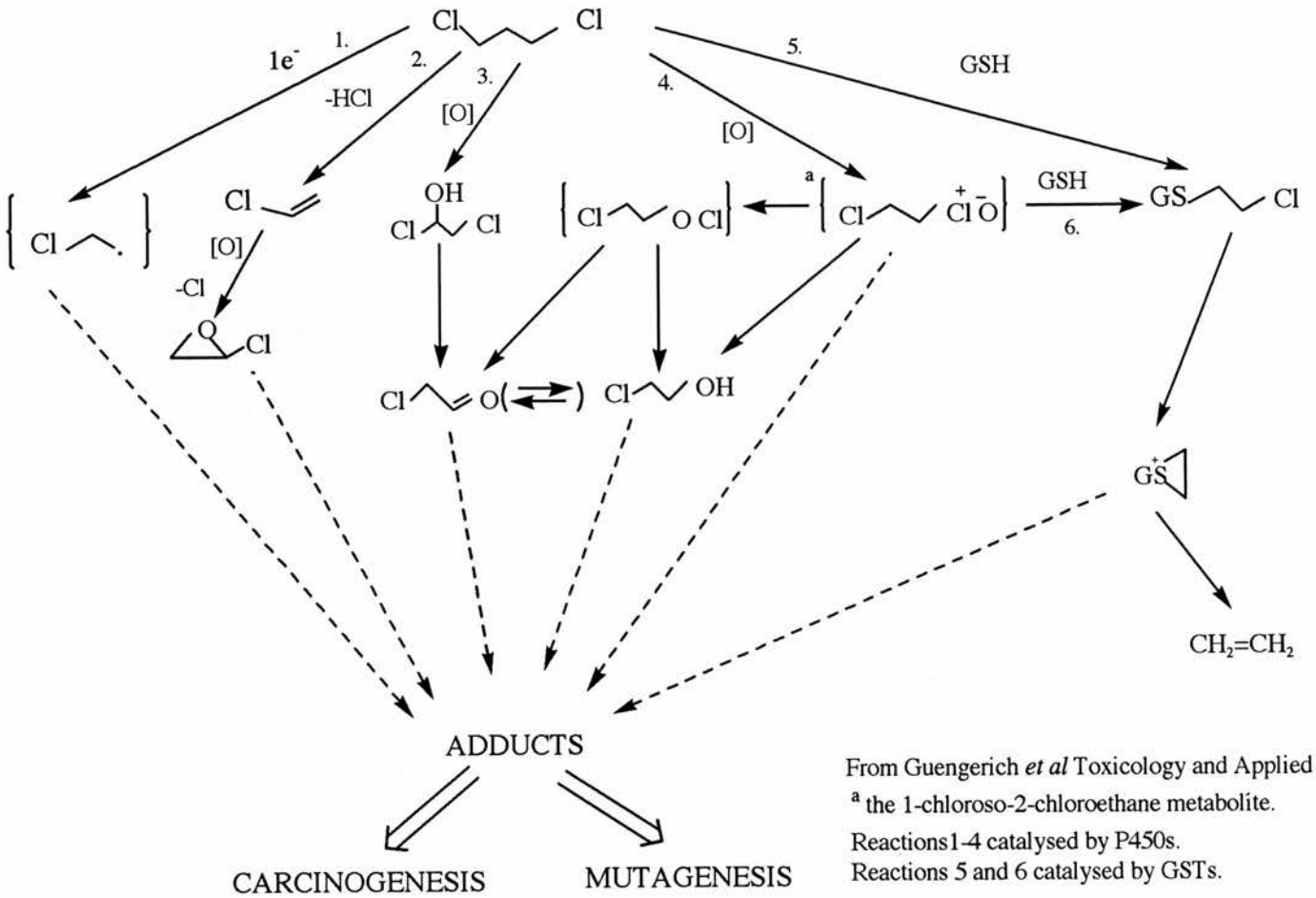
A number of possible routes of metabolism exist for this compound and are illustrated in Figure 5.3 (adapted from Guengerich *et al* 1980). About two thirds of P450 metabolism produces 1-chloroso-2-chloroethane. Such haloso compounds are extremely unstable and readily undergo rearrangement, hydrolysis or direct interaction with cellular macromolecules (see figure 5.3). The 1-chloroso-2-chloroethane may also react with GSH to give the episulphonium ion, identical to that produced on direct conjugation of DCE with GSH and it was calculated that the microsomal GST coupled to the P450 system contributes approximately 20-30% of the episulphonium ion production (Guengerich *et al* 1980). Also the microsomal GST may contribute to a minor portion of this species by direct conjugation with DCE.

A number of reports suggest that DBE can activate the microsomal GST (Botti *et al* 1982 Lundqvist and Morgenstern 1992b), it is not known whether it is the direct action of DBE or one of the reactive species produced during metabolism causes the activation. However, cytosolic activity was diminished by this process, which may alter the flux through the different pathways as time progresses (Botti *et al* 1982).

To extrapolate these observations to the yeast has its limitations. In addition the chloro groups are replaced by bromines, which may alter the relative stability of the metabolites. DBE is certainly toxic to yeast, but which pathway prevails is not known. The conditions for maximal expression from the PGK promoter are high glucose concentration and the later period of the growth phase, as discussed in the previous section these conditions at which endogenous P450 were found.

The expression of drug-metabolising enzymes in yeast provides a unique opportunity to test the hypothesis. Expression of various P450s

Figure 5.3 Postulated metabolism of 1,2-dichloroethane.



From Guengerich *et al* Toxicology and Applied Pharmacology **55**, 303-317 (1980)

[a] the 1-chloroso-2-chloroethane metabolite.

Reactions1-4 catalysed by P450s.

Reactions 5 and 6 catalysed by GSTs.

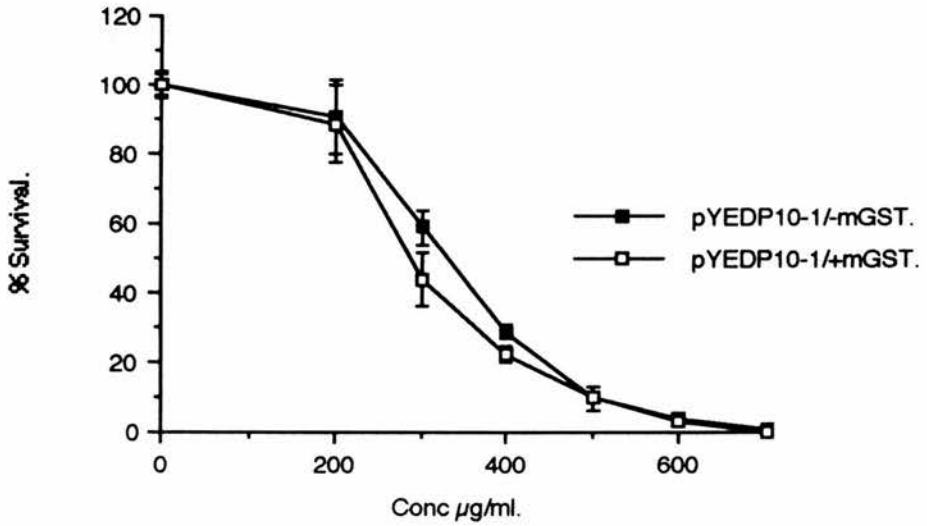
and the microsomal GST would enable such experiments to be carried out.

5.3.3 Chlorambucil.

The levels of cytosolic GST have been shown to be raised in tumours and cell lines treated with chlorambucil. Expression in yeast of human α and π class GST in *S.cerevisiae* offered protection against this compound compound. In addition, results suggested the resistance process was GSH-dependent (Black *et al* 1990). The expression of GSTs in mammalian cells gave more variable results (see table 4.1).

There are no reports of the microsomal GST protein being raised in cell lines (Wareing 1989) or in tumours made resistant to chlorambucil, or any other chemotherapeutic agent. Also the protein lacks inducibility by xenobiotics (Morgenstern *et al* 1982a). Probably as a result of these two observations, the involvement of the microsomal GST in the resistance to anti-tumour agents has not been rigorously studied. *In vitro* studies have shown the microsomal GST capable of a 2-5 fold increase in the amount of conjugate produced over the non-enzymatic value (Dulik *et al* 1990). In the light of prior findings the use of cytosolic enzymes might have been a more relevant choice to investigate the involvement of GSTs in chlorambucil-glutathione conjugation. Furthermore the lack of sequence homology and differences in enzymatic activities between the two forms of GST may imply different functions. These facts taken together make the use of the microsomal enzyme in the investigation of chlorambucil metabolism, as related to potential phenotypic resistance to this compound, a strange choice. The microsomal GST from cynomologous monkey liver was attached to cyanogen bromide activated Sepharose 4B. The conditions on the Sepharose 4B column would be unlikely to reflect the cellular environment, especially as the microsomal GST is found in the membrane. The metabolites analysed by reverse phase high performance liquid chromatography (HPLC). Results identified two glutathione conjugates of chlorambucil ; the mono and diglutathionyl forms.

Figure 5.4 Chlorambucil cytotoxicity experiment.



The data shown in the above graph is from single representative experiment. For each concentration the cultures were plated out in triplicate and from these results the standard deviation was calculated. The error bars shown in the graph are the standard deviations expressed as a percentage of the control value.

Chlorambucil is a bifunctional alkylating agent, it reacts with DNA to produce monoalkylation products or highly cytotoxic cross-links (di-alkylation) (Calabresi and Parks 1980). Chlorambucil needs no enzymic activation, but spontaneously forms an electrophilic carbonium ion that reacts with nucleophilic centres in DNA (Hemminki and Kalla 1986). Such an alkylating agent may well be a candidate for activating the microsomal GST, however no such study has ever been preformed.

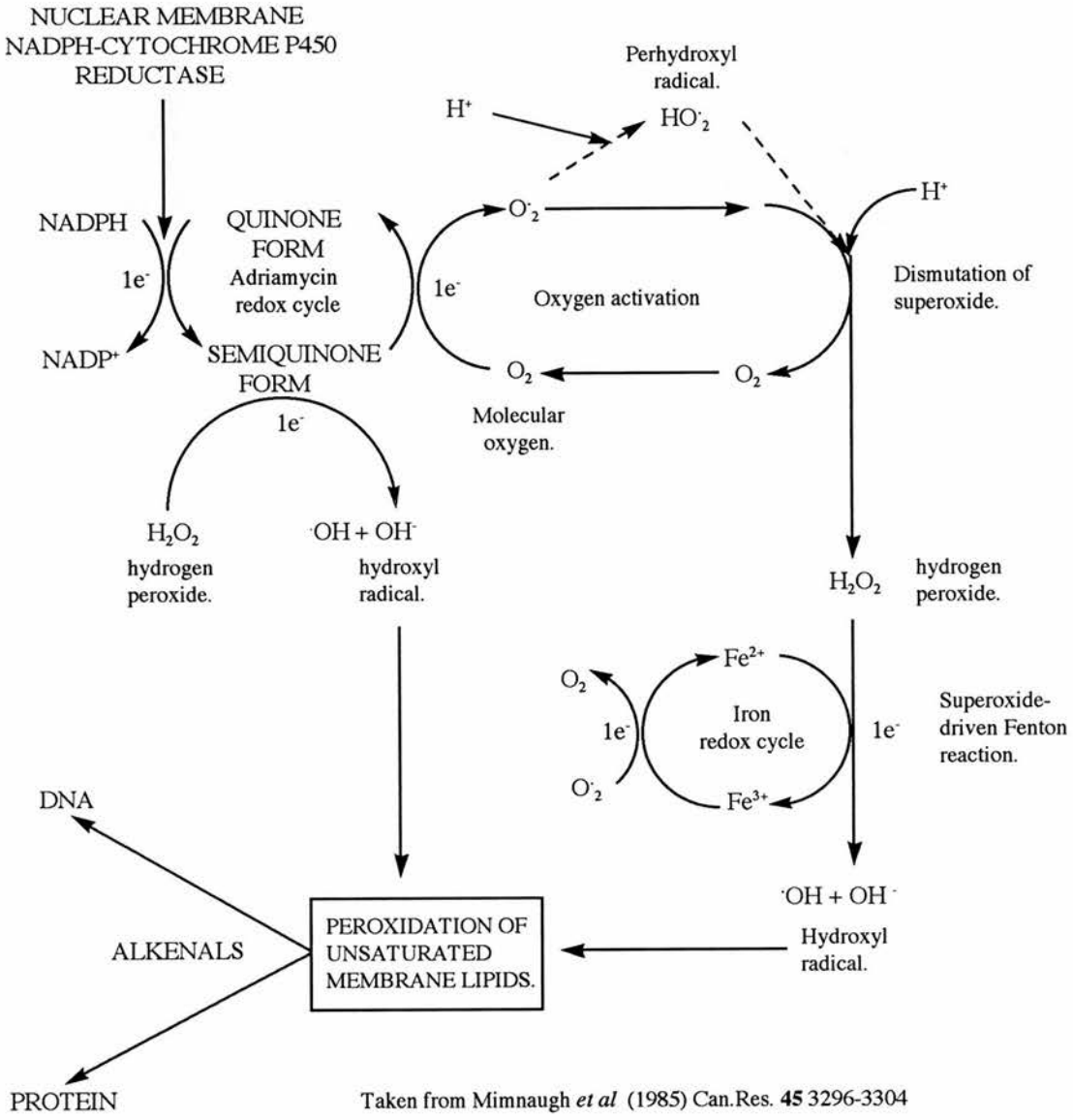
Figure 5.4 shows the data from a chlorambucil cytotoxicity experiment. Expression of the microsomal GST in yeast had no effect on the toxicity of chlorambucil. Chlorambucil is unstable in an aqueous environment, although its organic nature might suggest lipid solublility. With the purified protein under the experimental conditions described only a 2-5 fold increase over non enzymic rate was observed. In the expression system the level of expression and possible inactivity of some of the enzyme, may not be sufficient to afford protection to cells expressing the protein. The expression of the cytosolic enzymes in yeast caused a large increase in GST activity and increased resistance to this compound. The modest increases observed in the mammalian systems were accompanied by a more modest resistance to this compound, which suggests levels of expression have an effect on levels of resistance. In addition it was suggested GST expression might not be the only factor required to bring about resistance to this compound (Townsend *et al* 1991).

5.3.4. Adriamycin.

Adriamycin is an anthracycline antibiotic, these compounds have been used in the treatment of cancer since the late 1960s (Young *et al* 1981). Adriamycin produces a number of cytotoxic effects, such as DNA fragmentation by interaction with topoisomerase II (Tewey *et al* 1984) and interaction with membranes (Tritton and Lee 1982).

Adriamycin has the ability to undergo redox cycling between the quinone and semi-quinone form, see Figure 5.5. This does not happen spontaneously at physiological pH, but is dependent on P450 reductase activity (Bartoszek and Wolf 1992). The free radicals generated, namely

Figure 5.5 Schematic diagram of adriamycin-induced redox cycling.



Taken from Mimnaugh *et al* (1985) Can.Res. **45** 3296-3304

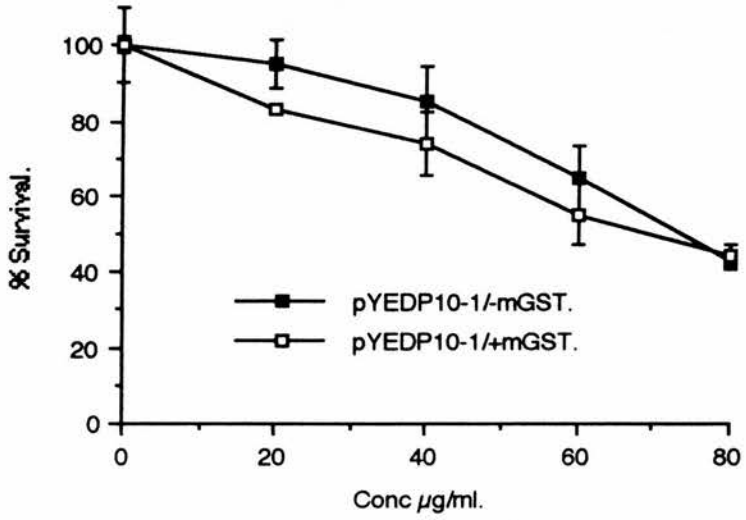
the superoxide and hydroxyl radical, are thought to be responsible for the observed lipid peroxidation (Mimnaugh *et al* 1985; Goodman and Hochstein 1977).

Several lines of evidence have demonstrated the importance of the glutathione redox cycle in removal of these species, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) an inhibitor of glutathione reductase greatly potentates the effect of adriamycin (Babson *et al* 1981). More recently a number of adriamycin-induced resistant cell lines have been characterised and show increased glutathione peroxidase activity (Sindra *et al* 1989; Samuels *et al* 1991). Alternatively an increase in pathways feeding in the GSH redox cycle are induced, such as the hexose monophosphate shunt (Yeh *et al* 1987). Eventually adriamycin toxicity causes glutathione depletion due to the action of the redox cycle (Kramer *et al* 1988). The microsomal GST has been shown to have peroxidase activity (Reedy *et al* 1980) and activity towards products of lipid peroxidation (Mosialou and Morgenstern 1989). Also the possibility that under conditions of oxidative stress the enzyme could be activated, were discussed in Section 1.4. As a result adriamycin was chosen as a means to test the ability of the microsomal GST to protect against lipid peroxidation.

Figure 5.6 shows a typical adriamycin cytotoxicity experiment. No difference was observed between cells expressing the microsomal GST and control. The mechanism of toxicity of adriamycin is diverse, but it would appear the microsomal GST cannot offer protection in this system.

The maintenance of membrane thiols correlates with cell viability under conditions of oxidative stress (Orrenius 1985). Addition of the membrane anti-oxidant α tocopherol offered protection against adriamycin toxicity (Pascoe *et al* 1989). The toxicity of *N*-acetyl-ρ-benzoquinione imine (NAPQI) and 3,5-dimethyl-*N*-acetyl-ρ benzaquinone imine (3,5-Me_2-NAPQI) were both shown to cause rapid depletion of GSH, again cell viability again correlated with loss of protein thiols. On analysis of individual protein thiol loss, susceptible protein were identified and the microsomal GST was such a protein. *In vitro* both compounds increased the microsomal activity (Weis *et al* 1992).

Figure 5.6 Adriamycin cytotoxicity experiment.



The data shown in the above graph is from single representative experiment. For each concentration the cultures were plated out in triplicate and from these results the standard deviation was calculated. The error bars shown in the graph are the standard deviations expressed as a percentage of the control value.

The organic peroxide, t-butylhydroperoxide also causes depletion of GSH, NADPH and release of GSSG from rat hepatocytes (Eklow *et al* 1984), however, as with adriamycin the effect was maximised on the addition of BCNU. This implicates the GSH redox cycle, involving direct reduction of t-butylhydroperoxide by glutathione peroxidase. Recent studies with isolated hepatocytes have shown the microsomal GST to be activated after exposure to this compound (Lundqvist and Morgenstern 1992b). Unlike the quinone compounds no reactive metabolites of t-butylhydro peroxide are produced, however the activating species has not been identified. Some debate in the literature as to what agent is capable of causing activation of this enzyme *in vivo*; the arguments have largely focused on GSSG and a number of alkylating agents (as discussed in Section 1.2). Whether the increase in microsomal GST as a result of thiol loss is a casual effect or has some significance in protection against oxidative stress has yet to be determined.

It is not known whether adriamycin treatment *in vivo* could cause an increase in the microsomal GST activity, but this would certainly be an interesting study. Potentially disruption of thiol status, or the production of reactive metabolites, or reactive oxygen species (ie H_2O_2, $O\cdot_2$ and $OH\cdot$), could all activate the enzyme and protein thiol loss would appear to be involved in adriamycin toxicity. All the afore mentioned studies have been preformed in mammalian systems. The effect of hydrogen peroxide has been studied in *S.cerevisiae* in two recent studies (Jamieson 1992; Collinson and Dawes 1992). Both studies showed that the yeast became resistant to oxidative insult in the later phases of growth. As the experiments described here were carried out at later growth points the expression of the microsomal GST may have been unable to contribute to the already existing protection mechanisms. No studies on investigating protein thiol loss in yeast have been undertaken, but the method described by Cotgreave *et al* (1988) would provide the means for such a study and assessing the thiol status of the expressed protein. The level of microsomal GST expression is substantially lower than found in the rat liver and the lack of activity has been discussed and maybe it is possible that insufficient enzyme is present for an effect to be observed.

In summary, within the confines of this system the expression of the microsomal GST did not protect against adriamycin toxicity in yeast. The resistance to this compound afforded by recombinant expression of the cytosolic GSTs is very system specific. It cannot be ruled out that increased expression of this enzyme in yeast or in a different system might exhibit an effect. In addition it maybe true that expression of the microsomal GST alone is not sufficient to protect against adriamycin toxicity in this system. In chapter 1 a number of other GSH dependent membrane protection mechanisms were discussed.

5.4. Summary.

The expression of the rat microsomal GST in *S.cerevisiae* caused an increase in sensitivity towards HCBD and DBE. The literature shows HCBD, but not DBE to be a specific substrate for the microsomal GST. However, data from similar studies with other GST isoenzymes do not reflect the specific activities observed *in vivo* . In the study described here involvement of the endogenous P450 system was implicated in drug activation, but no direct evidence exists. However if studies were extended to co-expression of various cytochromes P450 with the microsomal GST in this system, then these ideas could be fully investigated.

Chlorambucil has been shown to be conjugated with GSH in the presence of microsomal GST, but in the yeast system the level of activity obtained was not capable of affording protection against its cytotoxic effects. The low level of expression of the protein may have been responsible or simply GSH conjugation is not an effective detoxification mechanism for this compound, as suggest by some of the mammalian cell transfection systems.

Loss of membrane thiols is considered to be a critical step in the toxicity of a number of chemicals. The modification of the highly susceptible thiol in the microsomal GST results in an increase in enzyme activity and this may be a means of protecting membranes. Adriamycin is known to cause thiol depletion and lipid peroxidation and was chosen as a candidate to examine this putative role of the microsomal GST.

Expression of the protein in yeast did not alter the sensitivity towards the drug. To show that the microsomal GST does not function in this manner further studies would be needed. Firstly, it would be important to establish that adriamycin causes covalent modification of the microsomal GST in yeast or any other system for that matter. The use of the method developed by Cotagreve *et al* (1988), could be usefully applied in demonstrating this and overall thiol status within the yeast. The precise activating species for the microsomal GST is in many cases unknown, whether GSSG (or other oxidised thiols) produced during oxidative stress (Masukawa and Iwata 1986; Anyia and Anders 1989a; Lundqvist and Morgenstern 1992 b), or by hydrogen peroxide and reactive oxygen species (Anyia and Anders 1989b, 1992) or only alkylating species (Haenen *et al* 1988, Wallin *et al* 1991; Lundqvist and Morgenstern 1992 b) are capable of increasing activity is fiercely debated in the literature.

Treatment of the yeast system with adriamycin and BCNU would focus the attack on the thiol depletion aspect of adriamycin. There are a number of interesting candidates with subtly different properties such as menadione, t-butyl hydroperoxide, NAPQI and 3,5-Me_2NAPQI which were all discussed in chapter 1.

Further investigations of this nature coupled with assessment of thiol status would help to establish whether the thiol mediated activation of the microsomal GST is merely casual or has a significance in membrane protection.

Chapter 6: The expression of the rat microsomal glutathione S-transferase in *Escherichia coli.*

6.1 Introduction.

The heterologous expression of proteins in *Escherichia coli* (*E.coli*) is one of the oldest heterologous expression systems and as a result considerable lore has accumulated over the years. However, there are greater differences between mammalian cells and prokaryote, than between higher and lower eukaryotes, and this may present problems. As a generalisation, it is assumed that a protein can be expressed in *E.coli* as long as it is not too small, too large, too hydrophobic or does not contain too many cysteines (Goeddel 1991). Most generalisations have their limitations, and as with the yeast system discussed in the last chapter, the results are example-specific.

In Chapter 4, Section 4.1, the application of heterologous systems to the study of drug metabolising enzymes was discussed. This was exemplified by the use of mammalian and yeast systems to study the role of the cytosolic GSTs in drug resistance. Toxicological studies on the effect of xenobiotics in bacteria have employed the Ames test (Ames 1979) and this too has been adapted to incorporate heterologous protein expression. For example the expression of the human α class, B_1B_1, and π class GSTs in *Salmonella typhimurium* TA 1538 caused an increase in mutation rate when exposed to 1,2-dibromoethane and 1,2-dichloroethane implicating these enzymes in the toxicological activation of these compounds (Simula *et al* 1993). The use of a bacterial expression system for the cytosolic GSTs would seem appropriate given their soluble nature and lack of post-translational modification. However, the suitability of bacteria for expression of a membrane protein is not so obvious at the outset. Although bacteria are devoid of organelles found in the eukaryote systems, however a number of membrane proteins have been functionally expressed in bacteria. The rat P450 reductase reductase has been expressed in the *Salmonella* strain LR5000 and employed to investigate the role of this enzyme in the bioactivation of mitomycin C (Bligh *et al* 1992). The expression of P450 reductase was greatly enhanced by N-terminal fusion with the signal

sequence of the OmpA protein, a major outer membrane protein, allowing the P450 reductase to pass across the cytoplasmic membrane, where the signal peptide was removed and the protein accumulated in the periplasmic space (Shen *et al* 1989). Successful expression of a number of P450 enzymes has been achieved in bacterial systems (Porter and Larson 1990, Larson *et al* 1991 and Barnes *et al* 1991). Enhancement of expression of 17α hydroxylase was observed in *E.coli*, when the N-terminal region of the protein was altered. This was probably due to the adoption of a more favourable codon usage rather than alteration of the membrane nature of the protein (Barnes *et al* 1991).

The second use of heterologous expression systems has been to study various aspects of protein structure/function relationships by the application of site directed mutagenesis (SDM). In Chapter 1 analysis of the catalytic function of the cytosolic GSTs using this technique was discussed. Early chemical modification studies implicated a histidine residue in the deprotonation of glutathione (Aswathi *et al* 1987). However an elegant recombination experiment demonstrated otherwise. The rat liver GST 3-3 contains four histidine residues. These were replaced with [*ring* -2-^{13}C] histidine by growth of the bacteria, expressing GST 3-3, in a medium containing the labelled histidine. The purified expressed GST 3-3 was subjected to ^{13}C NMR allowing the histidine residues within the protein to be viewed spectroscopically. The titration of the four resonances were measured in the pH range 4-9, but the values remained the same in the presence and absence of glutathione. If a histidine had been involved in stabilising the thiolate ion then the pKa of the individual histidine would have altered in the presence of glutathione (Zhang *et al* 1991). Further evidence came from conservative mutation of the individual histidines to an arginine, which failed to significantly alter the catalytic activity of the protein (Wang *et al* 1991 and Zhang *et al* 1991). This example serves to demonstrate the versatility of such studies, the replacement of residues with amino acids synthesised with different isotopes has the advantage that no misleading confirmational changes are made. The residue responsible for interaction with the thiolate ion is tyrosine 7, as confirmed by the crystal structure (Rienemer *et al* 1991). This was also confirmed by SDM where by a

phenylalanine in position 7 lead to only 2-8% of wild type activity being present (Stenberg *et al* 1991).

Expression of mammalian proteins in prokaryotes, especially membrane forms, is not without potential problems. The differences in the initiation of translation consensus sequences between *S.cerevisiae* (Hamilton *et al* 1987) and mammalian (Kozak 1984) genes was discussed in Chapter 4, however these sequences show no relation to those found in bacteria. In bacteria there is a conserved sequence 5' of the ATG called the Shine and Dalgarno, or ribosome binding site (RBS), which shows a variable degree of complementation to a region close to the 3' end of the 16s ribosomal RNA (rRNA) (Balbas *et al* 1990). In general the RBS has four nucleotides from the sequence AGGAGG placed at a distance of between 5 to 9 base pairs, with 7 been average, from the ATG. The heterologous cDNA to be expressed must be manipulated to carry this sequence or the ATG be placed at the correct distance from the RBS in the vector, if present.

The sequence after the ATG is also considered important, GGAU and AAAA enhances translation due to a requirement for a relatively unstructured sequence, which is further illustrated by the removal of stem loop structures also enhancing translation (Balbas *et al* 1990). As mentioned earlier the alteration of the first 7 codons of the 17α hydroxylase P450 allowed translation of the protein, which had been previously inhibited in bacteria (Barnes *et al* 1991). The mutation in the second codon was from a tryptophan (TGG) to an alanine (GCT), which is the preferred second codon in the *lac* Z gene (Looman *et al* 1987), the third and fourth codons were changed to TTA, a silent mutation which increased the number of adenosines and uridines, as discussed above. Also the last nucleotide in codons 6 and 7 were silently mutated to a adenosine (GCT → GC**A**) and thymidine (GTC → GT**T**), respectively in order to minimise RNA secondary structure (Schauder and M^{C}Carthy 1989). These changes allowed the successful expression of functionally active P450 incorporated into the bacterial membrane, which was previously was not translated.

Another P450, rabbit P450IIE1 was successfully expressed in *E.coli* (Larson *et al* 1991). The cytoplasmic domain of the protein is thought to be bound to the membrane of the endoplasmic reticulum by one or two

transmembrane segments at the N-terminus (residues 3-23 and 32-48). The removal of the first segment caused only a slight reduction in the amount of protein associated with the membrane with a concomitant increase in the amount found in the cytoplasm. The exact nature of the membrane interaction of the P450 is not known, although these results would appear to support a helical-hairpin hypothesis. However, other membrane-interactions exist with the cytoplasmic domain cannot be ruled out, or an alternative model is in operation, where as many as 10 membrane transmembrane spanning domains have been proposed. Interestingly, removal of this N-terminal region resulted in the overall level of P450IIE1 expression been reduced by a third. The authors offered decrease in protein stability and/or inefficiency in protein translation as possible explanations.

The environment of the *E.coli* is potentially hostile to foreign proteins for a number of reasons. For examples; the reducing environment present in bacteria does not permit cysteine rich proteins to form the required disulfide bonds in order fold properly (Geoddel 1990) Many strategies have been developed to overcome this problem, such as engineering the protein to be secreted into a different environment or refolding the protein *in vitro*. The bacteria themselves have their own devices for dealing with foreign proteins, proteolysis of these proteins acting as a primitive immune system. However the mechanisms by which *E.coli* recognises "self" are not understood (Gold 1990).

The problems encountered are, as always, example-specific and the strategies adopted to overcome them will be discussed in the context of the work.

6.2 Aims of the chapter.

The work in the *S.cerevisiae* system yielded some interesting findings, concerning the toxicological role of the rat microsomal GST (Chapter 5). However the limitations of the system were centred around the low levels of protein and the apparent lack of activity. Chapter 4 describes steps taken to try to increase these parameters, but none of these were successful in significantly raising protein expression.

By expressing the rat microsomal GST in a different system, namely *E.Coli*, higher levels of recombinant protein might be achieved. Although the microsomal GST is located in the mitochondria and endoplasmic reticulum in mammalian cells (Morgenstern *et al* 1984), the successful expression of a number of membrane proteins has been achieved in this system and examples have been cited in the previous section.

The purpose of setting up the *E.Coli* system was two-fold. Firstly, the Ames (mutagenicity) test and a cytotoxicity test were available to investigate the role of the rat microsomal GST in the metabolism of a range of compounds. Secondly, to carry out SDM experiments to investigate the activation and catalytic mechanisms of this enzyme.

6.3 Results and discussion.

6.3.1 The expression of the rat microsomal glutathione S-transferase in *E. coli* under the control of the *tac* promoter.

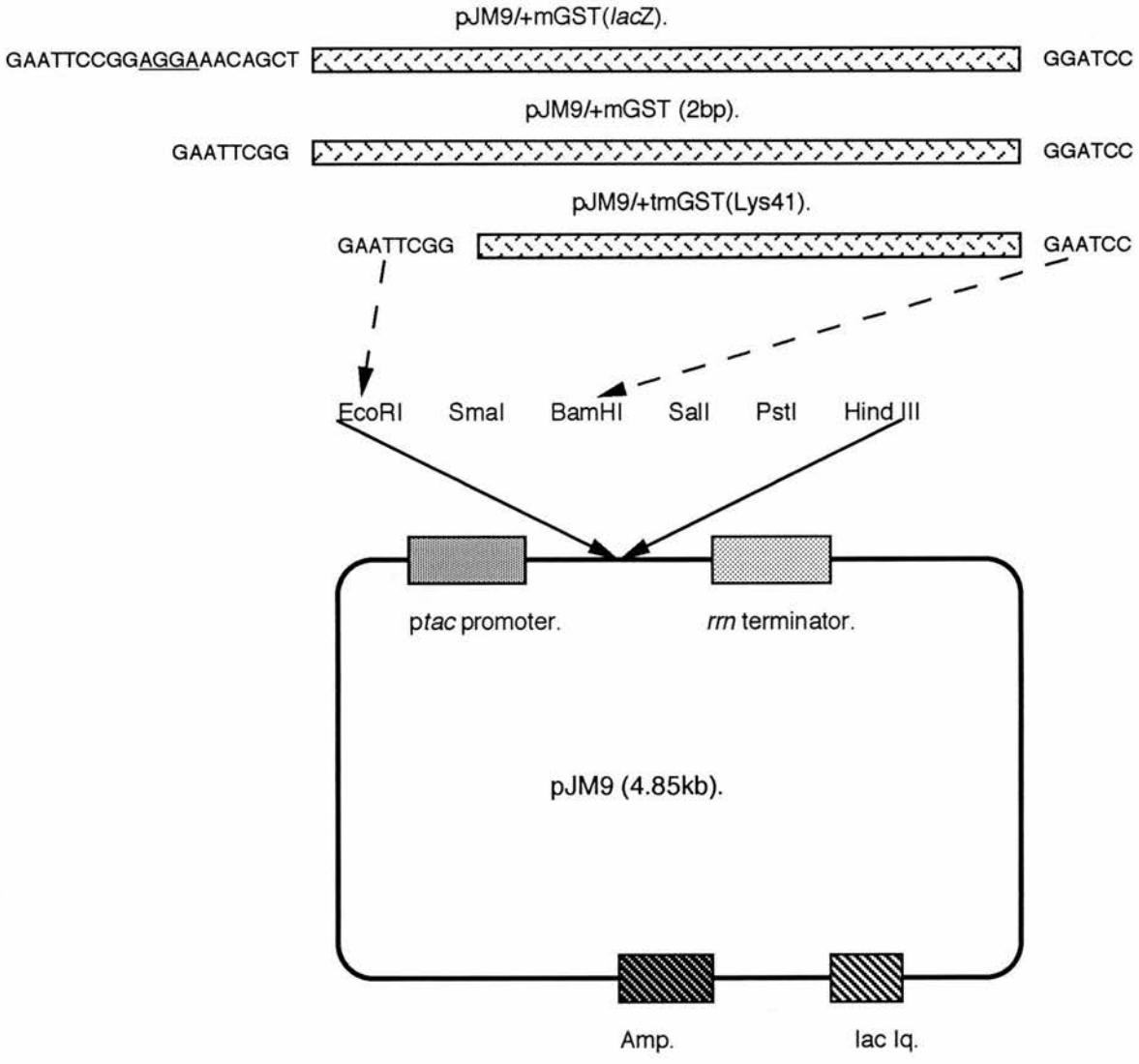
Figure 6.1 shows the expression vector and the 5' non-coding sequences of a number of microsomal GST cDNAs generated by PCR for use in this vector. The 5' regions were altered either to incorporate a RBS or to place the ATG at an acceptable distance from the one downstream of the *tac* promoter in the vector (de Boer *et al* 1983). The use of the *Eco* R1 site in the vector is acceptable, in this respect, and a clone with 2 nucleotides between the *Eco* R1 and ATG is illustrated. A second clone was generated using the 11bp prior to the ATG of the *lac* Z gene which contain the AGGA Shine and Delgrano sequence at the optimum distance from the ATG. In order to avoid the potential problems of expressing membrane proteins in this system, a truncated clone of the microsomal GST starting in the coding region 3' of lysine 41 was created. The rationale behind removing the membrane domain at this point was discussed in Section 4.4.8. In addition, the experiments with P450IIE1 expression in *E. coli* demonstrated that removal of a membrane spanning domain from this protein allowed the expression of catalytically active protein (Larson *et al* 1991).

The vector pJM9 was constructed from pKK223.3 (Pharmacia) and contains the *tac* promoter, which is a hybrid of the *lac* and *trp* promoters (de Boer *et al* 1983). This promoter will be repressed in bacterial strains containing the *lac* I^q gene, due to the presence of *lac* UV5 sequences in the promoter, the repression is removed by addition of isopropyl βD-thiogalactoside (IPTG).

The controls in these experiments are the bacteria transformed with the parental plasmids. All the bacteria are grown overnight, then inoculated into fresh media at a dilution of 1:20, grown for a further hour and then IPTG was added at a final concentration of 1mM. The cultures were incubated for a further 5 hours before harvesting and preparation for analysis.

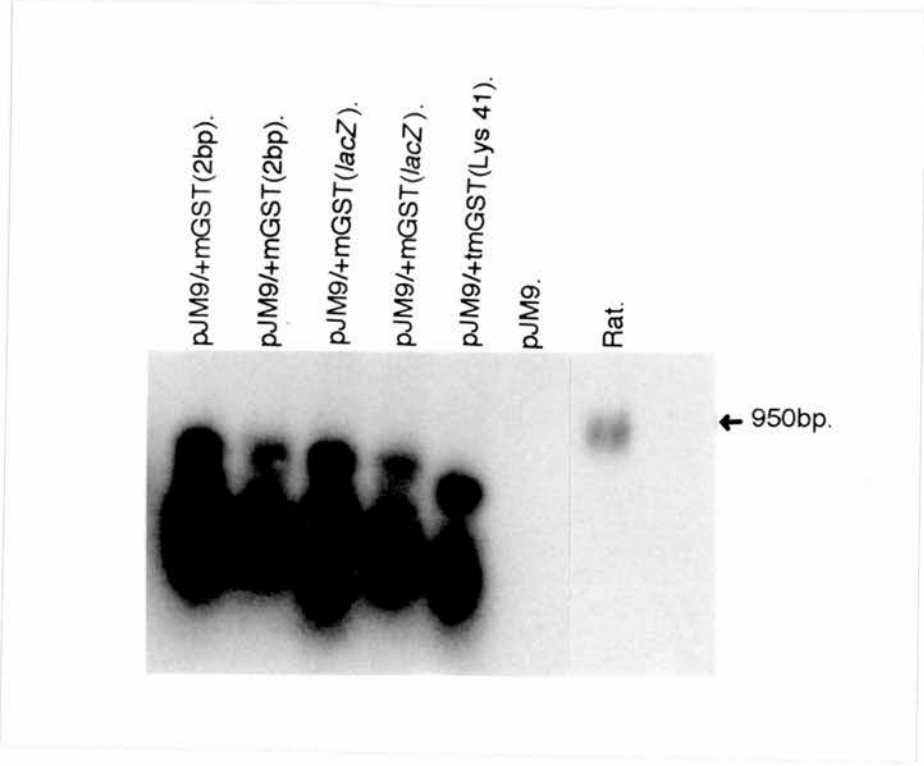
Figure 6.2 shows the Northern blot analysis of the constructs described. Constructs containing microsomal GST-derived cDNAs all clearly

Figure 6.1 pJM9 Expression vector and cloning strategy.



AGGA is the *lac*Z ribosomal binding site.

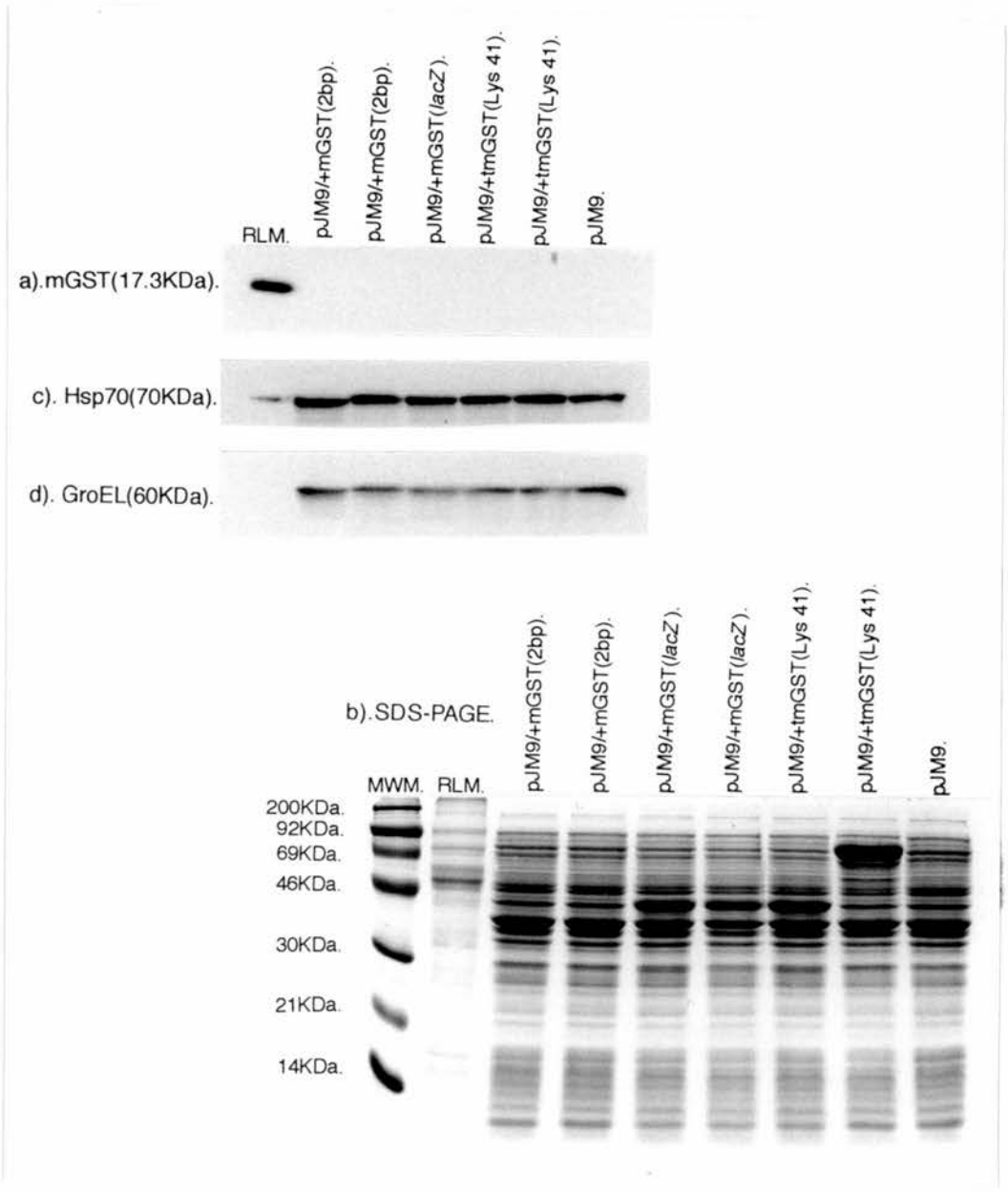
Figure 6.2. Northern blot analysis of bacteria transformed a range of rat microsomal glutathione S-transferase cloned into the pJM9 expression vector.



10 μg of total RNA loaded.

Details of the expression constructs are given in the text and Figure 6.1.

Figure 6.3. Analysis of bacteria transformed a range of rat microsomal glutathione S-transferase cloned into the pJM9 expression vector.



For Western blot analysis (Figures a,c and d): 10μg of protein from bacterial cell extracts loaded.

For SDS-PAGE analysis (Figure b): 30μg of protein from bacterial cell extracts loaded.

RLM: rat liver microsomal protein.

MWM: molecular weight markers.

produce mRNA that hybridised with the rat microsomal GST cDNA probe. However, no rat microsomal GST protein was detectable in these samples with Western blot analysis using the anti-rat microsomal GST antibody, shown in Figure 6.3a. On examination of the coomassie blue stained SDS PAGE (Figure 6.3b), it can be seen that several proteins seem to be induced in some of the samples. The approximate molecular weights of these proteins were approximately 70KDa and 40KDa. The two stress response proteins Hsp70 and GroEL/Hsp 60 have molecular weights 70KDa and 60KDa, respectively. A heat shock response has been reported in *E.coli* , when expressing abnormal proteins (Goff and Goldberg 1985). The samples were then subjected to Western blot analysis using antisera raised against bacterial Hsp70 and GroEL, but no increase in either of these proteins was observed (Figure 6.3c and d, respectively). Both these proteins are molecular chaperones thought to be involved in polypeptde folding and assembly in bacteria (Gatenby *et al* 1990), and hence possible overexpression during foreign protein expression. However the fact that these bands do not cross-react with Hsp70 and GroEL antisera does not exclude them from being other stress proteins induced by the production of an unfavourable protein.

The absence of any microsomal GST suggests that either the protein is unstable in the bacterial environment or the mRNA is untranslatable. Proteolysis was described as the method by which bacteria remove foreign proteins from their system in Section 6.1. To investigate this possibility, a time course experiments were performed on transformants containing full length and truncated microsomal GST cDNAs. However, not only was the growth of expressing transformants unaffected with respect to the control, but no microsomal GST protein was detectable at any point in the growth curve (data not shown). This suggests the mRNA is untranslatable, or the protein is turned over immediately it is translated.

6.3.2 Expression of the rat microsomal glutathione S-transferase in *E. coli* using a secretion system.

In the introduction to this Chapter the enhanced expression of P450 reductase was described using a system that directed the protein to the

Figure 6.4 pIN-III-*omp*A expression vector and cloning strategy employed to insert the rat microsomal GST.

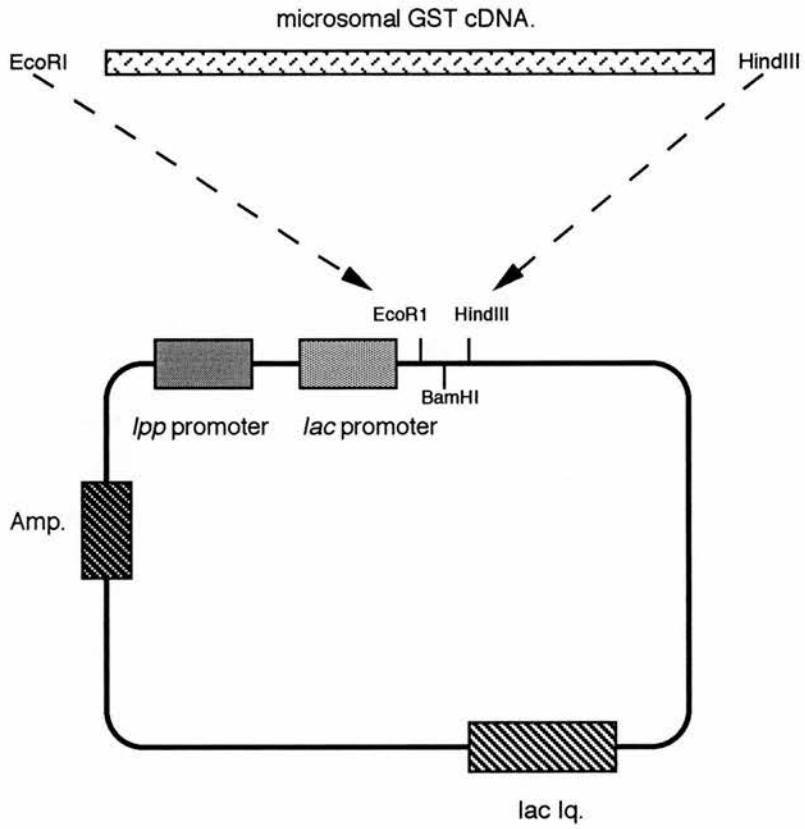
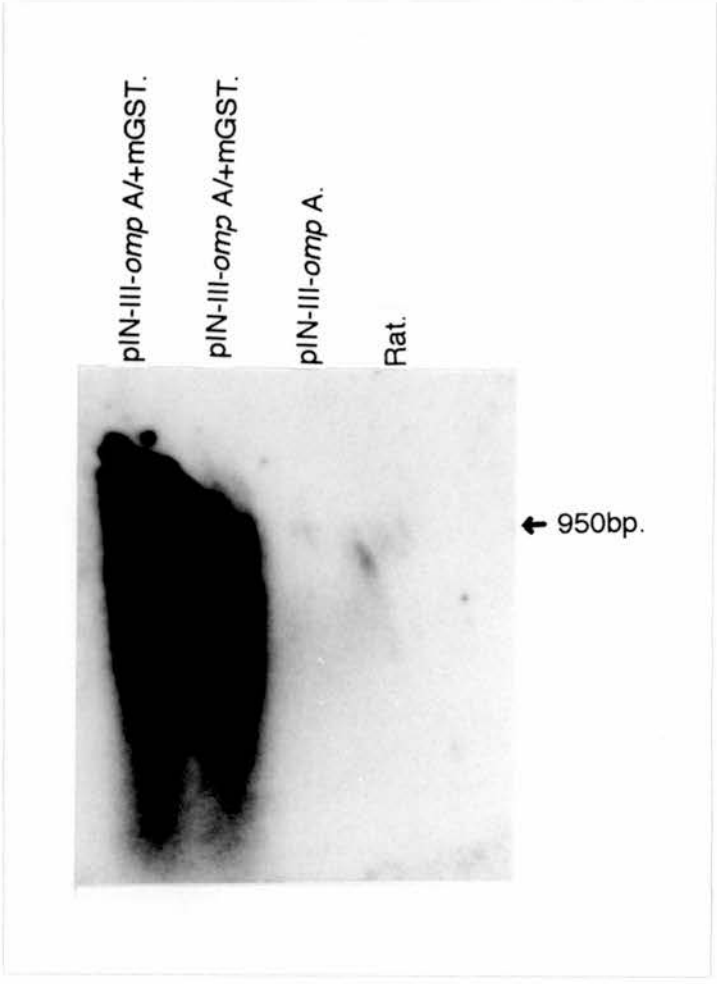


Figure 6.5. Northern blot analysis of bacteria transformed with the pIN-III-*omp*A expression vector containing the rat microsomal glutathione S-transferase and the parental vector.



10 μg RNA loaded.

periplasmic space (Shen *et al* 1989). The protease activity of the periplasmic space is considered to be less than the cytosol, and a number of proteins such as nucleases and proteases have been expressed in this region of the cell, which would be toxic if expressed in the cytoplasm (Ghrayeb *et al* 1984). In addition the OmpA protein signal sequence will contain a favourable codon usage for the initiation of translation.

Figure 6.4 shows the pIN-III-*omp*A expression vector (Ghrayeb *et al* 1984) and the cloning strategy employed to insert the rat microsomal GST cDNA. The vector contains both the *lpp* promoter and the *lac* promoter, the latter allowing induction of expression with IPTG. Figure 6.5 shows Northern blot analysis, using the rat microsomal GST cDNA probe, and again mRNA is produced by these constructs. However, samples of bacteria were taken over a 6 hour period, and analysis by Western blot revealed no microsomal GST protein to be present.

Any problems of initiation of translation should have been overcome by the insertion of the OmpA signal sequence. However it is impossible to say whether the protein was degraded in or en route to the periplasmic space, or indeed if it was produced at all.

6.3.3 Coexpression of the rat microsomal glutathione S-transferase with the *arg* U gene in *E.coli.*

The previous results suggest that some form of translational problem may be preventing production of the microsomal GST in bacteria, or that severe proteolysis preventing its accumulation. Proteins which are not readily expressed in bacteria have a high content of rare codons, in their genes, which can potentially limit the rate of translation. One such codon is the arginine AGA/AGG triplet and a number of examples have been cited (Brinkman *et al* 1989). The results demonstrated an inverse relationship between the number of AGA/AGG codons and the level of protein expression, and in addition, the production of such proteins inhibits growth. When the percentage of AGA/AGG codons rose above 3% a dramatic reduction of protein production was observed. The rarity of the AGA/AGG codon is reflected in the availability of the $tRNA^{Arg}_{AGA/AGG}$. However, co-transfection with the gene encoding this tRNA, the *arg* U gene (Lindsay *et al*

Figure 6.6. The arginine codon usage in the rat microsomal glutathione S-transferase.

```
                                                  10                                                  20
M  A  D  L  K  Q  L  M  D  N  E  V  L  M  A  F  T  S  Y  A  T  I  I  L  A  K  M  M  F
ATG GCT GAC CTC AAG CAG CTC ATG GAC AAC GAG GTG TTG ATG GCT TTT ACC TCC TAT GCA ACG ATC ATT CTT GCC AAG ATG ATG TTC

      30                                                  40                                                  50
L  S  S  A  T  A  F  Q  R  L  T  N  K  V  F  A  N  P  E  D  C  A  G  F  G  K  G  E
CTG AGC TCC GCG ACT GCA TTC CAG AGG CTA ACC AAC AAG GTT TTT GCC AAC CCG GAA GAC TGT GCT GGC TTC GGC AAG GGG GAG

            60                                                  70                                                  80
N  A  K  K  F  L  R  T  D  E  K  V  E  R  V  R  R  A  H  L  N  D  L  E  N  I  V  P
AAT GCC AAG AAG TTC CTT CGG ACT GAC GAG AAG GTG GAA CGC GTG CGA AGA GCC CAC CTG AAT GAC CTT GAA AAC ATC GTT CCC

         90                                                  100                                                 110
F  L  G  I  G  L  L  Y  S  L  S  G  P  D  L  S  T  A  L  I  H  F  R  I  F  V  G  A
TTT CTC GGT ATC GGC CTC CTG TAC TCC CTG AGC GGA CCG GAT CTC TCT ACA GCC CTC ATT CAC TTC AGA ATC TTT GTG GGC GCT

      120                                                 130                                                 140
R  I  Y  H  T  I  A  Y  L  T  P  L  P  Q  P  N  R  G  L  A  F  F  V  G  Y  G  V  T
CGG ATC TAC CAC ACC ATT GCT TAC TTG ACT CCC CTT CCT CAG CCA AAC AGG GGC TTG GCA TTT TTT GTT GGC TAC GGA GTT ACT

                  150
L  S  M  A  Y  R  L  L  R  S  R  L  Y  L  *
TTG TCA ATG GCT TAC AGG CTG CTC AGG AGC AGA CTG TAC TTG TAA
```

AGA and AAG are rare codons in *Escherichia Coli.*

% unfavourable bias = [AGA+AGG] / [XXX]

Full length microsomal GST = 4.5%
Truncated (at lysine 41) mGST = 5.31%

1989), caused an increase in protein levels and alleviation of growth inhibition (Brinkman *et al* 1989). Interestingly, the *arg* U gene product is thought to have a role in cell replication (Garcia *et al* 1986), which would explain the inhibition of growth in bacteria expressing genes with a high AGA/AGG content.

Figure 6.6 shows the coding region of the rat microsomal GST with the arginines residues and codon usage indicated. The percentage of AGA/AGG is 4.5% for the full length microsomal GST and 5.3% for the truncated form. The *arg* U gene was available on a kanamycin selectable plasmid, pUBS520, with an IPTG-inducible promoter and compatibility with pBR322-derived expression vectors (gift from Professor R.Mattes). However cotransfection, using double antibiotic selection, of this plasmid with the expression constructs described in Sections 6.3.1 and 6.3.2, failed to result in the production of any microsomal GST. The problems regarding the expression of the microsomal GST in bacteria are therefore not exactly analogous to those observed by Brinkman *et al* (1989). No inhibition of growth was observed with bacteria carrying the expression construct and no protein production was observed. This suggests that although the high representation of the AGA/AGG codon in the rat microsomal GST may be a factor in the translation of the protein, other forces must also be important.

6.3.4 Use of the T7 RNA polymerase to direct expression of the microsomal glutathione S-transferase in *E. coli.*

The bacteriophage T7 promoter is immensely powerful and in conjunction with the T7 RNA polymerases can cause rampant transcription of genes placed under its control. Not only is the strength of the promoter crucial but the selectivity of the system important in directing gene expression. The T7 promoter and termination signals are rare in *E.coli* DNA and are only recognised by the T7 RNA polymerase. This protein is 5 times more active than the *E.coli* RNA polymerase and is resistant to the RNA polymerase inhibitor rifampcin. This means that almost all the transcription in the cell will be driven by the T7 RNA polymerase, when present, thereby allowing the expression of a number of gene products which cannot be expressed by more conventional means (the system is reviewed by Studier

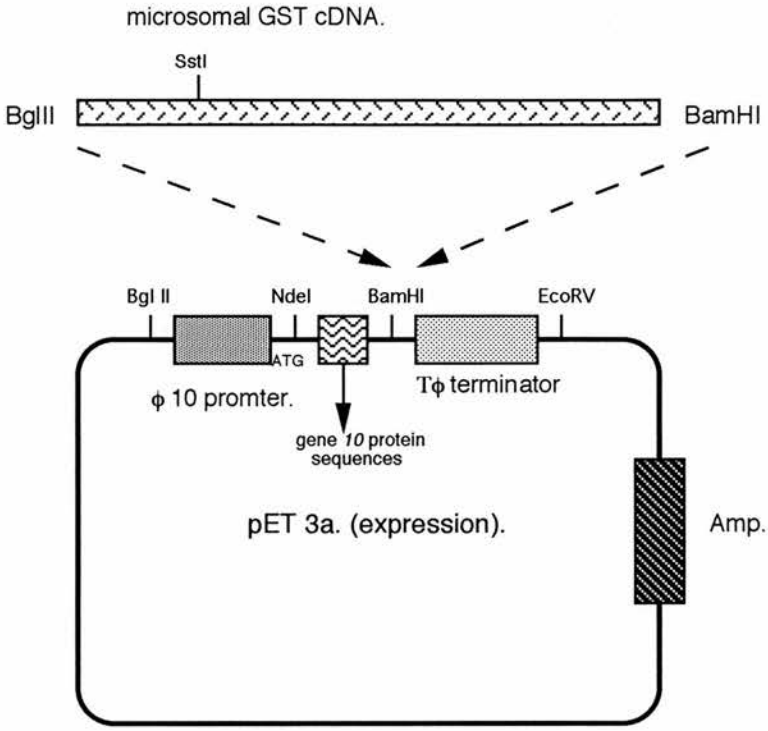
et al 1990).

The pET vectors (plasmid for expression by T7 RNA polymerase) contain the T7 promoter, which is derived from the ϕ *10* promoter, one of the six strong promoters derived from T7 DNA. The following downstream nucleotides, when transcribed, allow a 8bp stem and loop structure which is thought to confer RNA stability and facilitate translation. Downstream of the ϕ *10* promoter is a small portion (-23 to +96 relative to the mRNA start) of the gene *10* protein, which is the major capsid protein of T7. The coding region of the protein allows the first 11 amino acids of the gene *10* protein to be expressed as a fusion with the protein to be expressed, which is cloned into a unique *Bam*H1 site (a series of vectors exist with the *Bam*H1 site in all three reading frames). Fusion with the gene *10* protein will allow the efficient initiation of translation of the recombinant protein. Alternatively, the cDNA to be expressed can be cloned into a *Nde* 1 site encompassing the ATG of the gene *10* protein. A T7 transcription terminator Tϕ is found downstream of the *Bam*H1 site and insures efficient termination of transcription by the T7 RNA polymerase.

The T7 RNA polymerase gene is under the control of the *lac* UV5 promoter and is present, along with the *lac* I^q gene, on a λ derivative of phage 21- the so called DE3 bacteriophage. The DE3 lysogen has been incorporated into the chromosome of BL21pLysS, then designated BL21(DE3)pLysS The pLysS refers to the chloramphenicol selectable plasmid in this strain that carries the T7 lysosyme gene. T7 lysozyme is a bifunctional protein which not only cleaves the proteoglycan bacterial cell wall, but also inhibits T7 RNA polymerase. In this system, the T7 lysozyme will inhibit the T7 polymerase and prevent production of potentially toxic genes under the control of the T7 promoter. However, the level of T7 polymerase will be greatly increased following IPTG induction and the inhibition by the T7 lysozyme will no longer be effective. The level of T7 lysosyme is not sufficient to be harmful to the cell, but will make the cells readily lysable. BL21 strains of *E.coli* are also deficient in the *lon* and *Omp* T proteases, the latter has been shown to cleave T7 RNA polymerase.

Figure 6.7 shows the pET 3a vector into which the cDNA for the rat microsomal GST was cloned at the *Bam*H1 site. Again the cDNA was

Figure 6.7 pET3 and pET3a expression vectors.

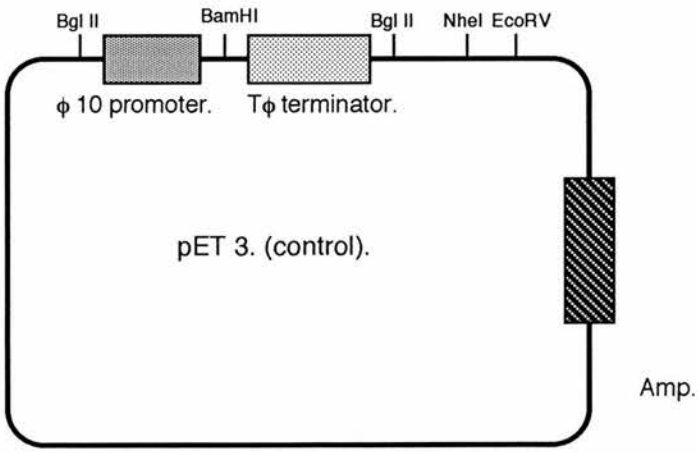


in order to clone the cDNA into the *BamH* 1 site the following oligomers were used to generate the cDNA with suitable restiction sites, as shown above.

5' oligomer: GA AGA TCT ATG GCT GAC CTC AAG CAG CTC ATG AAC

3' oligomer: AG GGA TCC TTA CAA GTA CAG TCT GCT CCT GAG CAG

The 2 nucleotides present 5' and 3' of the restiction sites (underlined) of the 5' and 3' oligomers, respectively, were inserted to facilitate restiction enzyme digest after PCR. All other nucleotides coresponded to the 5' and 3' sequences of the rat microsomal GST (DeJong *et al* 1988). Cloning the cDNA into the *Bam* H 1 site produced a fusion protein with the gene 10 protein product.



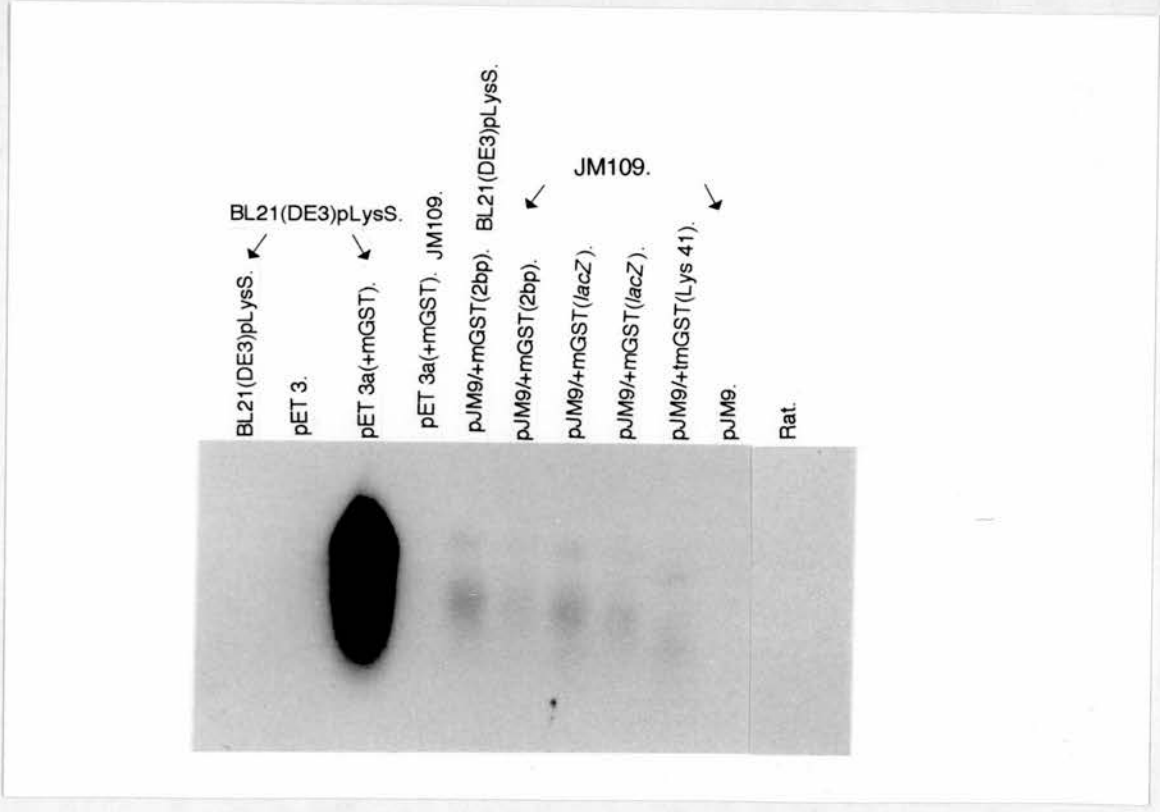
derived by PCR with a *Bgl* II site at the 5' and *Bam*H1 site at the 3' end of the cDNA. Once cloned into the vector the *Bgl* II site 5; of the ATG was destroyed, so the cDNA had to be removed by the *Bgl* II upstream of the promoter and by the *Bam*H1 site 3' of the TAA in the cDNA. The isolated fragment was cleaved at a unique *Sst* 1 site in the cDNA to allow directional cloning into M13 mp18/19 and was sequenced, to insure no mutations were introduced by the PCR.

Figure 6.8 shows Northern blot analysis of BL21(DE3)pLysS transformed with the control plasmid, pET 3, and the expression construct described above. Samples from the other constructs described in Sections 6.3.1 and 6.3.2 are also included. This Northern blot represents a different exposure to that in figure 6.2, where the pET transformants were not shown. However at this exposure, which is much shorter than in Figure 6.2, the mRNA from the earlier constructs are barely visible. The mRNA produced under the control of the T7 promoter is abundant in comparison and demonstrates the strength of this promoter in comparison to the others.

Figure 6.9 shows a time course graph of the bacteria transfected with the control plasmid and the expression construct. It can be seen there is a reduction in growth rate of the bacteria transformed with the expression construct. The coomassie blue-stained SDS PAGE gel loaded with whole cell samples taken at different time points shows the appearance of a 20KDa protein after induction with IPTG (1mM) (Figure 6.10c b). Western blot analysis of an equivalent gel (Figure 6.10a), using the anti-rat microsomal GST antibody, demonstrated this protein to be the recombinant rat microsomal GST and the observed increase in size was due to the gene *10* protein sequences. The control bacteria transformed with pET 3 were also analysed in the same manner and the western blot of these samples is shown in Figure 6.10b.

Figure 6.11 shows the effect of IPTG concentration on the expression of the rat microsomal GST under the control of the T7 promoter. As can be seen a small amount of protein is present before the addition of IPTG, but the level of expression is greatly increased on its addition. However, the concentration of IPTG did not affect the level of protein expression over the range tested. As a result further experiments used 0.1mM IPTG.

Figure 6.8. Northern blot analysis of bacteria transformed with rat microsomal glutathione S-transferase derived pJM9 and pET 3a expression constructs.



10 μg total RNA loaded.

Figure 6.9 Growth curve of BL21pLysS transformed with pET3 and pET3a(+mGST).

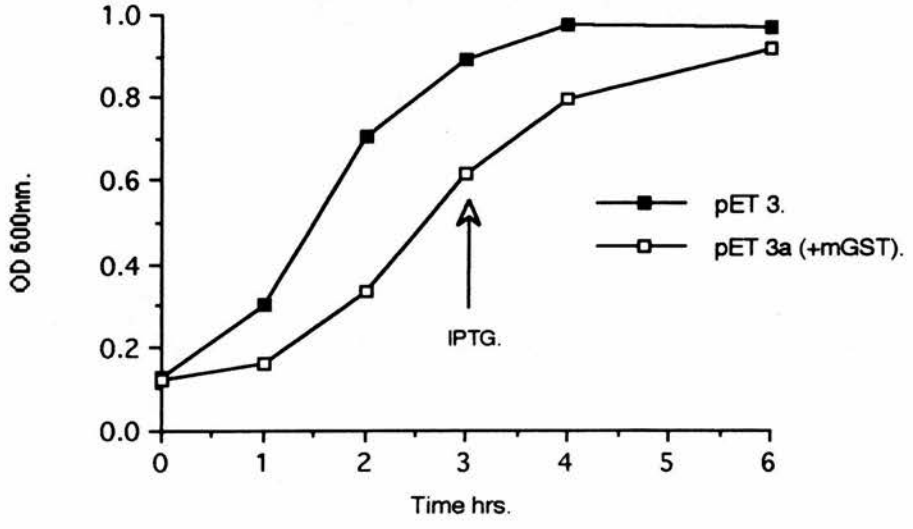
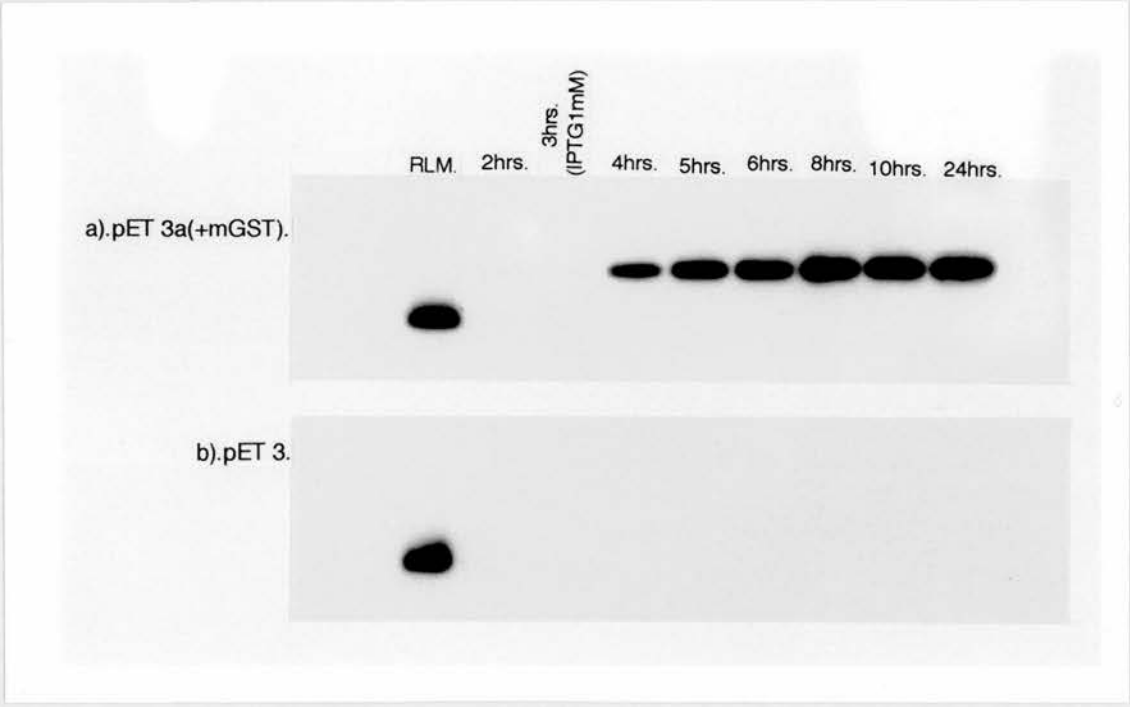
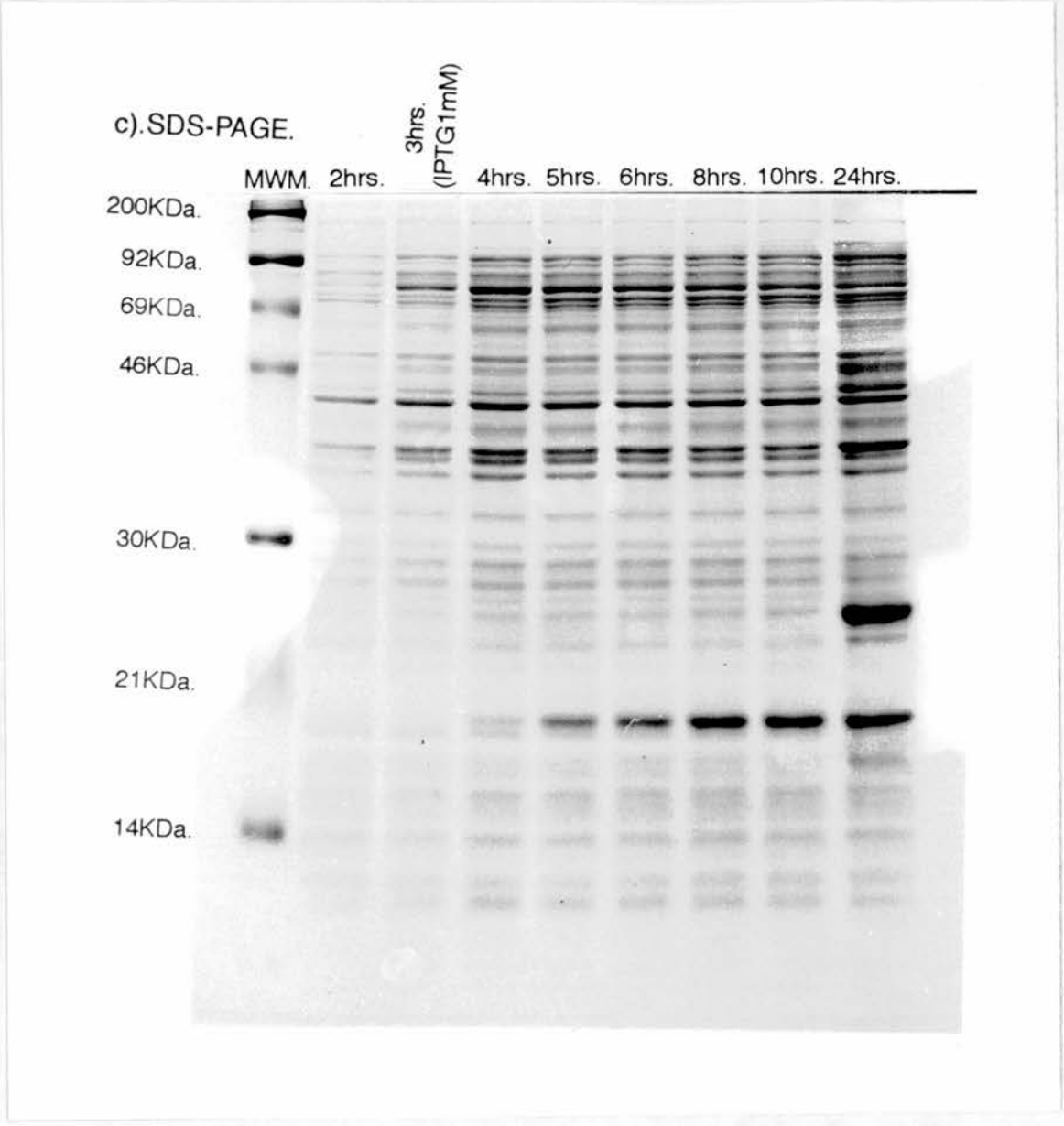


Figure 6.10. Time course of bacteria expressing the rat microsomal glutathione S-transferase under the control of the T7 system and control bacteria transformed with pET3 (Western blot analysis).



RLM: 5μg of rat liver microsomal protein loaded.
10μg of protein from whole cell bacterial extract loaded

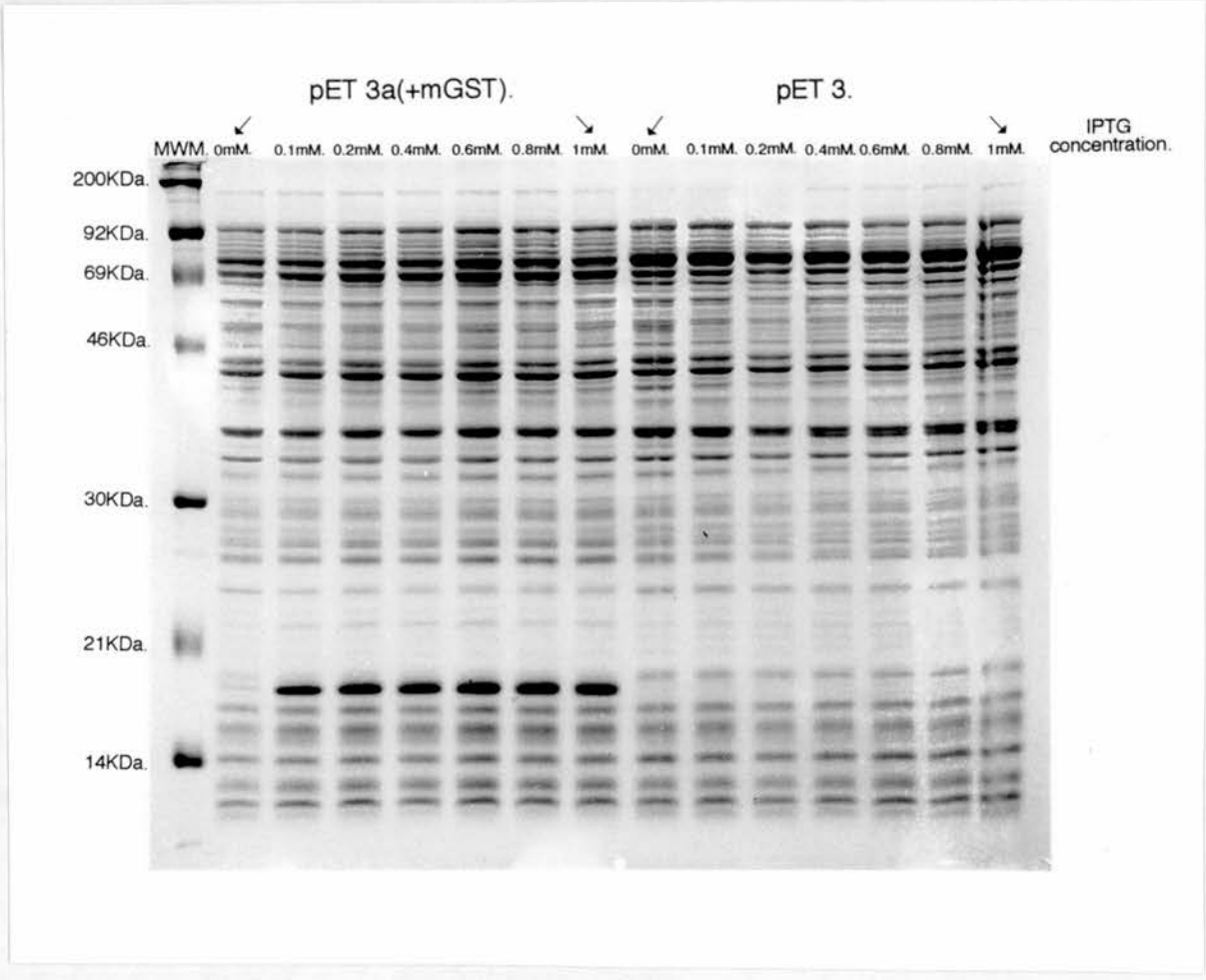
Figure 6.10. Time course of bacteria expressing the rat microsomal glutathione S-transferase under the control of the T7 system and control bacteria transformed with pET3 (SDS-PAGE analysis).



MWM: molecular weight markers.

30μg of protein from whole cell bacterial extract loaded.

Figure 6.11. The effect of IPTG concentration on the expression of the rat microsomal glutathione S-transferase under the control of the T7 system.



MWM: molecular weight markers.

30μg of protein from whole cell bacterial extract loaded.

6.3.5. Recovery of the active microsomal glutathione S-transferase expressed in *E.coli* under the control of the T7 system.

Despite the high levels of recombinant microsomal GST protein produced in this system, no activity could be measured. The cell pellet of the bacteria expressing the microsomal GST was white and remained opaque after sonication, whereas the control cells were yellow in colour and translucent after sonication. Furthermore bright bodies were visible at the ends of the bacterial cells and a large number of filamentous bodies could be viewed down the light microscope. These two observations are indicative of inclusion body formation, and would account for the lack of activity.

Inclusion bodies are aggregates of insoluble protein which have precipitated in the bacterial cytoplasm. There is no direct evidence to suggest why recombinant proteins should be sequestered into inclusion bodies (Marston 1986). It is not simply a response to the over expression of a foreign protein, which does not fold properly in the bacterial environment, as over expression of some *E.coli* proteins results in the formation of inclusion bodies. It may then seem reasonable to assume that if a protein is produced at such a rate that the cell's degradation system becomes saturated, then inclusion bodies may form. However, this does not explain why proteins such as prochymosin are insoluble, although expressed at low levels (Schoemaker *et al* 1985). Although the major constituent is the recombinant protein other constituents have been identified, such as the four subunits of RNA polymerase, some combination of the outer membrane subunits OmpC, OmpF and OmpA, 16s and 23s rRNA and nicked plasmid DNA, but no significance has been attached to these constituents (Schein 1989).

The best explanation here is that the microsomal GST is extremely unstable in bacteria an will only be expressed when the synthesis of the protein exceeds its turnover and in an insoluble form. Inclusion bodies are in fact resistant to degradation (Chen *et al* 1981). However, low levels of microsomal GST were observed before the addition of IPTG (Figure 6.10a), but no activity towards CDNB could be measured. In addition growing the bacteria in the absence of IPTG to a later time point (5hrs), in order a crude

membrane preparation could be performed, did not result in the detection of any activity, but under these conditions no protein was detected using Western blot analysis.

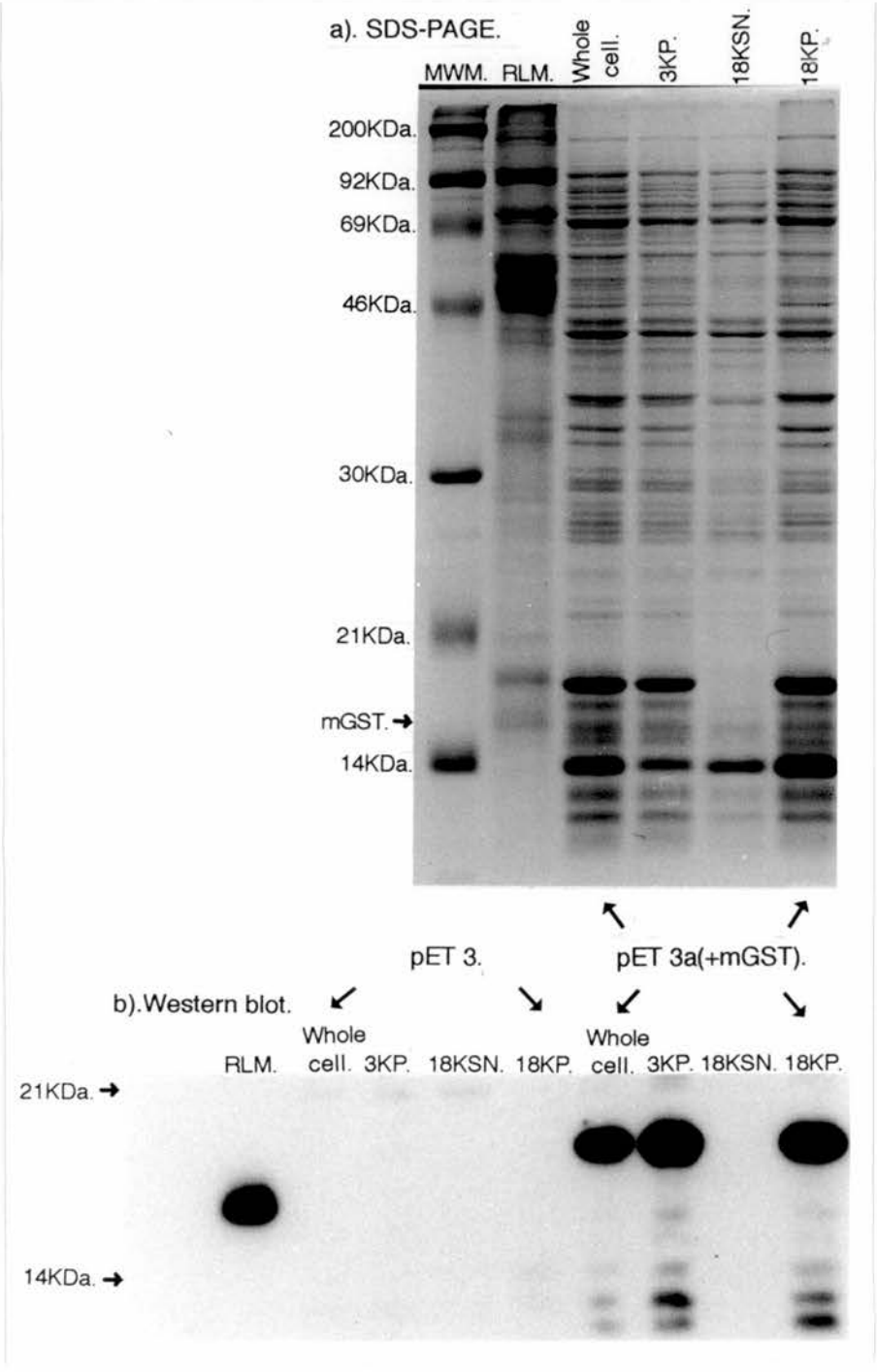
The loss of expressed protein to inclusion bodies is not an uncommon problem and the literature is full of methodology on the subject. Although some success has been achieved in this area, yields of active protein are generally poor and the results example-specific (Kohno *et al* 1990). The broad strategy is as follows after purification of the inclusion bodies from the bacteria, a powerful choatropic agent is used to solubilise the denatured protein, then a partial purification of the protein is performed and finally the denaturant is removed to allow the protein to refold in a favourable environment (Kohno *et al* 1990 and Marston 1986).

The inclusion bodies were purified in a crude form by differential centrifugation. Treatment with lysozyme was followed by sonication and the debris removed by a 3000g five minute spin. The supernatant was then spun at 18000g for 20 minutes to sediment the inclusion bodies. As can be seen from the Western blot in Figure 6.12b, the microsomal GST was located in the 18000g pellet (and in the cell debris) and the whole cell. The equivalent SDS-PAGE is shown in Figure 6.12a.

It was possible that some of the protein was in fact incorporated into the bacterial membranes, which would sediment in the 18000g pellet. To investigate this the pellet was resuspended in a phosphate based buffer containing 1% Triton X-100 and stirred at 4°C with samples being taken at various time intervals. The native protein is solubilised from rat liver microsomes by using 1% Triton X-100 in a phosphate based buffer (Morgenstern *et al* 1982). However, this treatment of inclusion bodies did not produce any activity towards CDNB. Analysis of the samples by SDS-PAGE were hard to interpret due to the presence of the Triton X-100, but no appreciable protein appears to have been released.

From these observations, it was assumed all the protein was insoluble. Two chaotropic agents were used to solubilise the inclusion bodies, 8M urea and 8M guanidine hydrochloride (Gn-HCl) and both were effective. Figure 6.13 shows the results of inclusion body preparations treated with phosphate buffer and phosphate buffer plus 8M urea. After treatment, and a further centrifugation, a sample of the supernatant and

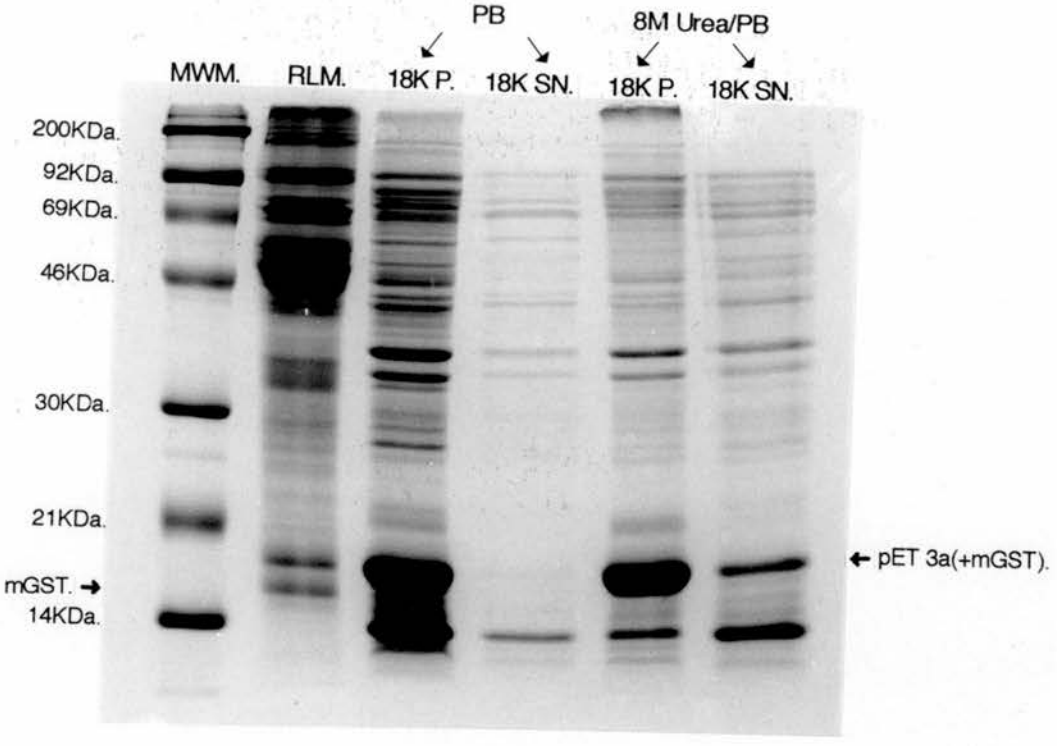
Figure 6.12. The localisation of the rat microsomal glutathione S-transferase expressed under the control of the T7 system within centrifugal fractions.



For a) RLM: 10μg of rat liver microsomal protein loaded.
MWM: molecular weight markers.
30μg of protein from centrifugal fractions loaded.

For b) RLM: 50μg of rat liver microsomal protein loaded.
10μg of protein from centrifugal fractions loaded.

Figure 6.13. Solubilisation of the rat microsomal glutathione S-transferase contained in inclusion bodies and expressed under the control of the T7 system, with 8M urea.



RLM: 10μg of rat liver microsomal protein.

MWM: molecular weight markers.

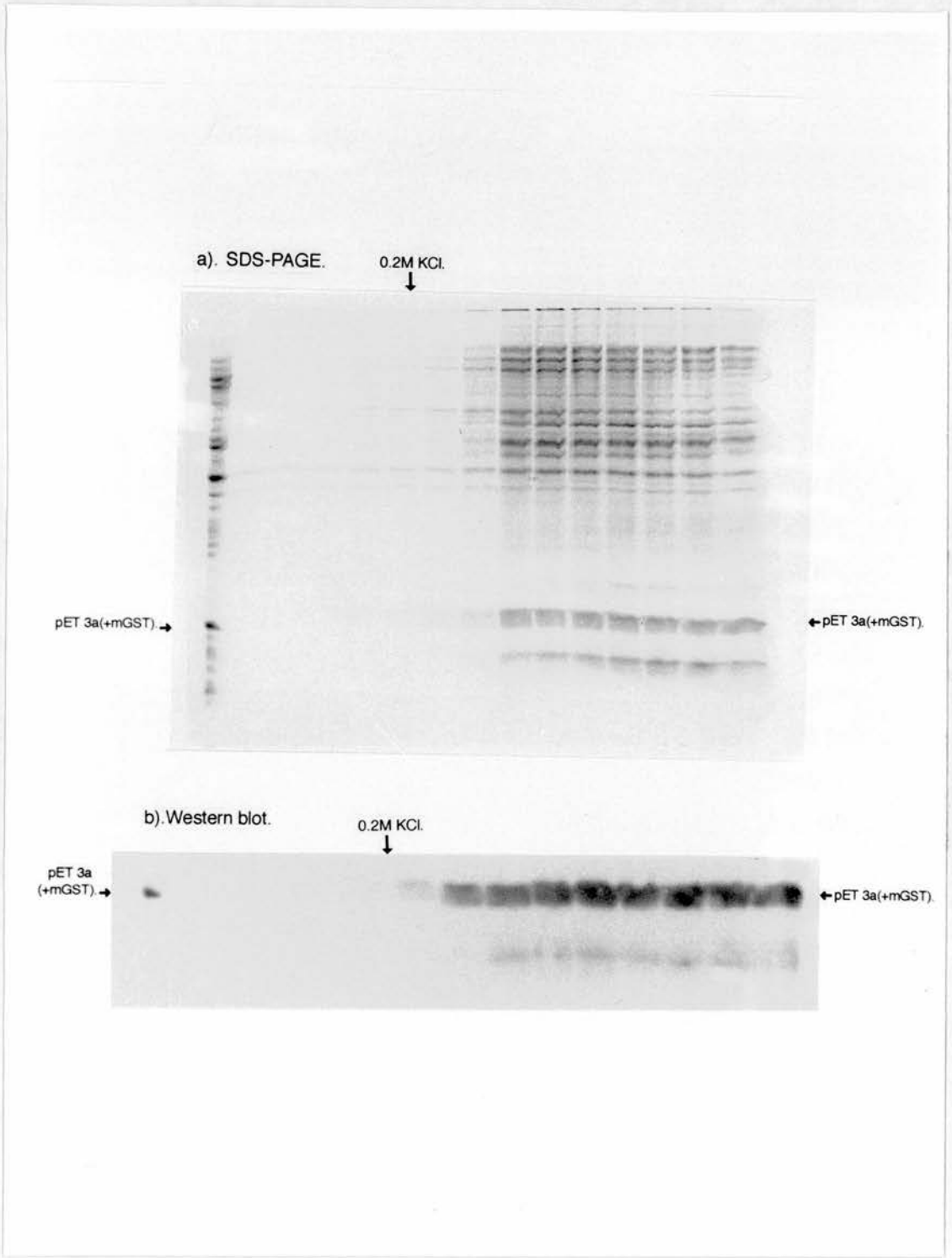
PB: phoshate buffer.

~30μg of total protein loaded.

pellet were run on SDS-PAGE. Appreciable amounts of the microsomal GST were released into the supernatant following solubilisation with urea, but not with phosphate buffer alone.

The purification of the microsomal GST from inclusion bodies was based on the purification procedure used to prepare the native protein from rat liver microsomes (Morgenstern *et al* 1982). The standard buffer contained 10mM potassium phosphate pH 7.0; 1% Triton X-100; 0.1mM EDTA, and 20% glycerol to stabilise the protein; GSH (1mM) was used as an antioxidant and to stabilise the GST. The native enzyme has been shown to be stable and active in this buffer (Morgenstern *et al* 1982). The inclusion body preparation was solubilised overnight in the standard buffer containing 8M Gn-HCl, this being preferable to using urea, because the Gn-HCl remained in solution at 4°C. After a 10000g spin to remove insoluble material, the concentration of Gn-HCl was reduced to 6M by the addition of the standard buffer. The material was then passed down a Sephadex G50 column in order to partially resolve the material and the fractions containing the microsomal GST were determined by SDS-PAGE. The resolution of the material was not particularly effective and some material precipitated during the procedure. The samples containing the highest concentration of microsomal GST were pooled and gradually diluted with sample buffer to a final Gn-HCl concentration of 0.6M and then dialysed to remove the last of the Gn-HCl. However, removal of the denaturant caused a great deal of material to precipitate, and this material was removed by a 10000g centrifugation. The concentration of the protein was low at this point and no activity could be measured. By applying the solution to a carboxy-methyl Sepharose column it was hoped that the microsomal GST would be concentrated on the column. Due to the low protein concentration the material was eluted with 0.2M potassium chloride, rather than a linear gradient of 0-0.2 potassium chloride as described by Morgenstern *et al* (1982). Figure 6.14 a and b show the coomassie blue stained SDS-PAGE and the Western blot analysis (respectively), using the anti rat microsomal GST antibody, of 100μl samples from fractions eluted from the CM-Sepharose column by 0.2 potassium chloride. Although the protein bands are smeared, due to the presence of Triton X-100, the recombinant protein is visible both on the SDS-PAGE and the Western blot. The addition of

Figure 6.14. Recovery of soluble rat microsomal glutathione S-transferase from inclusion bodies using CM-Sepharose ion exchange.



100µl of protein from the CM Sepharose column.
Standard: 30µg (SDS-PAGE) 10µg (b Western blot) of protein from bacteria whole cell extracts expressing microsomal GST.

potassium chloride failed to elute the protein in a sharp peak. However the lysozyme which was present in high concentrations, due to its use in the preparation, did elute from the column in a sharp peak indicating the column was functioning properly. These observations suggest that the hydrophobic nature of the microsomal GST caused it to interact with the column matrix and not to be concentrated by this process. However despite the visible presence of the microsomal GST there was no detectable activity towards CDNB at any of the stages after the removal of Gn-HCl.

The explanations for the lack of activity are numerous and include the possibility that the protein failed to fold correctly or assemble into an active trimer after the removal of Gn-HCl. Gn-HCl is a powerful chaotrope and may well have irreversibly damaged the protein. A cationic surfactant, cetyltrimethylammonium chloride (CTAC), was used in preference to urea and Gn-HCl to recover pig growth hormone from inclusion bodies (Puri *et al* 1992). Although the efficiency of solubilisation using CTAC was less than the two chatropes, the recovery of active protein was considerably higher being in the order CTAC> urea>> Gn-HCl. The superior refolding efficiency of the CTAC over the chatropes was explained by the lessening of damage to the growth hormone during solubilisation, also the low recovery and potential irreversible modification to protein structure during the use of urea and Gn-HCl has been reported by others (Marston 1986, Schien 1989 and 1990).

It is impossible to say which of these explanations, if any, are responsible for the inactivation of the microsomal GST. The use of a different solubilising agent, such as CTAC, might be instructive. Especially as this agent can easily be removed by ion-exchange chromatography, which is also used in the purification of the microsomal GST. Although this expression system could not be employed in an *in vivo* context ie in conjunction with the Ames test, it was hoped that sufficient quantities of recombinant protein could be prepared to allow the studies into catalytic and active processes.

6.3.6 Use of the recombinant rat microsomal glutathione S-transferase from the T7 expression system for raising an antibody.

Although no active protein could be recovered from inclusion bodies, the protein in an inactive form could be usefully employed to prepare antisera against the rat microsomal GST. One of the problems associated with the detection of heterologous protein expression in micro-organisms is the high degree of non-specific cross-reactivity of host proteins and antisera. This is because the animals in which the proteins are raised are continually exposed to micro-organisms in their environment, thereby invoking an immune response to these haptens. The non-specific cross-reactivity of the rat microsomal GST antibody in yeast was discussed in Section 2.7.5, however at this time insufficient sera was available for immunopurification.

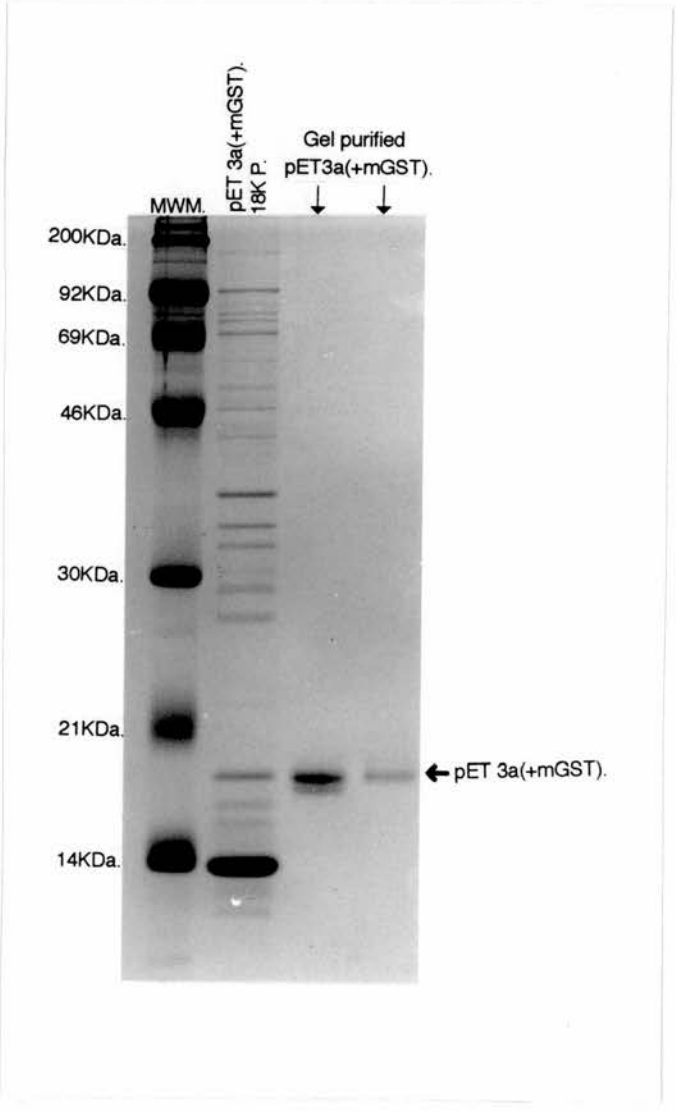
The recombinant microsomal GST was resolved from the other bacterial protein on SDS-PAGE. The band was excised and electro-eluted from the acrylamide. After concentration of the sample the protein was ready for injection into rabbits in order to raise antisera. This procedure was carried out at the Imperial Cancer Research Fund Laboratories at Clare Hall, London. Figure 5.13 shows a sample (10μl) of two such preparations loaded onto a 12% SDS-PAGE and stained with coomassie blue. In the first sample a faint band is observed below the recombinant microsomal GST.

Figure 6.15 shows Western blot analysis of samples of:- rat microsomes; *E.coli* expressing the rat microsomal GST under the T7 promoter (pET3a/+mGST); and the rat microsomal GST expressed in *S.cerevisiae* under the control of the PGK promoter(pYEDP10-1/+mGST.). The controls were included from the heterologous expression systems. The Western blot was carried out in triplicate and probed with 1) preimmune sera: Figure 6.16a 2) antisera raised against the recombinant microsomal GST prepared in this section: Figure 6.16b 3) the antisera raised against the purified rat microsomal GST (McLellan *et al* 1989): Figure 6.16c. The preimmune sera, Figure 6.16a, shows a strong cross-reacting band at ~10KDa in bacteria and ~30-40KDa in yeast, but nothing in rat liver microsomes.

The antisera raised against the recombinant protein recognises the

native protein and the rat microsomal GST expressed in yeast and bacteria. In addition a bacterial protein is recognised in the same region as the T7 directed microsomal GST. This can be explained by contamination of the samples injected into the rabbits with bacterial protein and also explains the origin of the second band in Figure 6.16. This limits the use of this anti-body in this form, for use with samples derived from bacteria expression. However, immunopurification of the antisera would eliminate this problem. The use of this antiserum, raised against recombinant microsomal GST, gave a very clean western blot with rat liver microsomes. This indicates a specific nature of this antibody in the analysis of rat tissue.

Figure 6.15.Purification of pET3a(+mGST) from SDS-PAGE for the preparation of antisera.

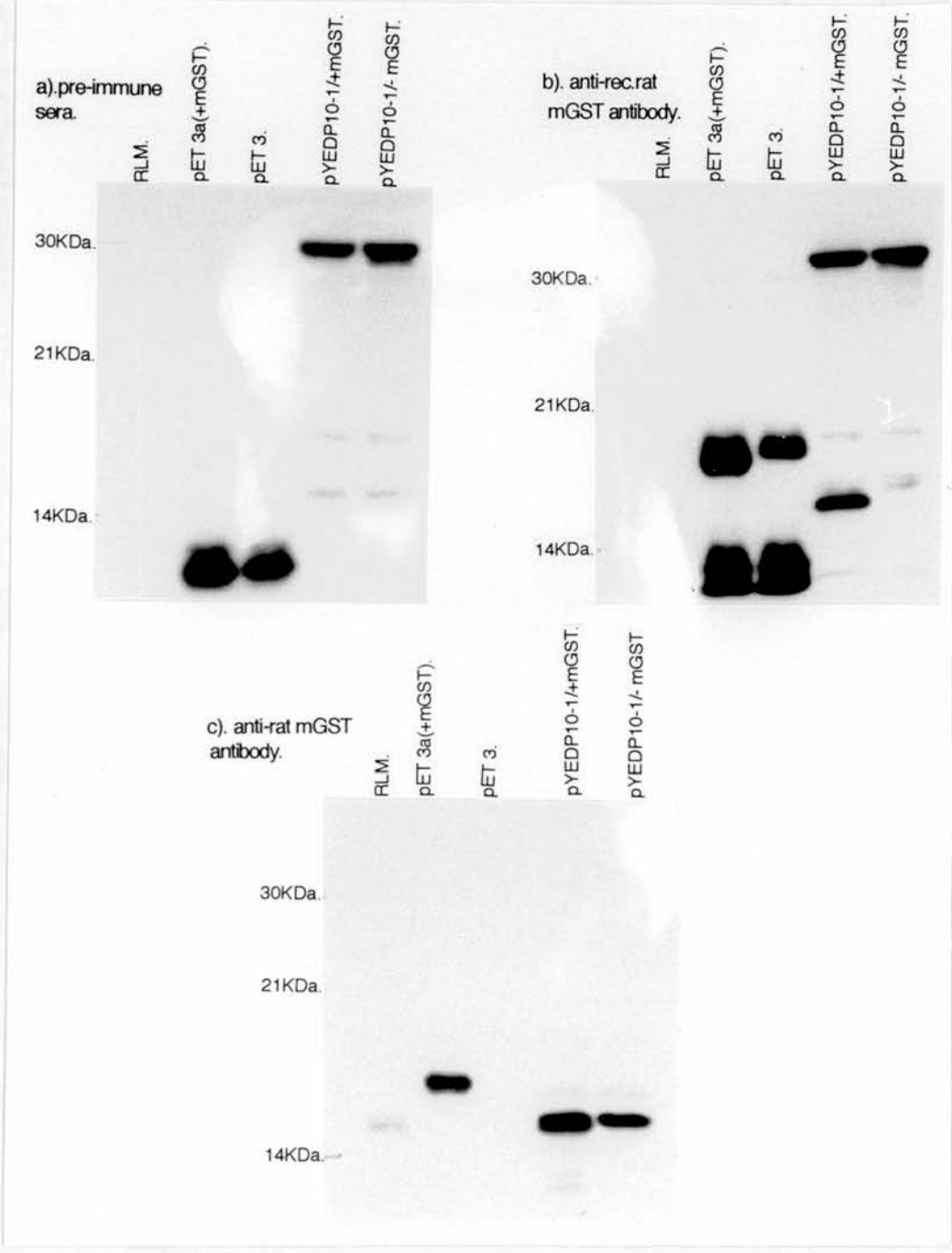


MWM: molecular weight markers.

10μl of gel purified pET3a(+mGST) loaded.

10μg from the 18K pellet loaded.

Figure 6.16. Western blot analysis of antisera raised against pET3a(+mGST) purified from bacteria.



RLM: 5μg of rat microsomal protein loaded.

10μg of protein from whole cell extracts from bacteria transformed with pET3a(+mGST) and pET3 were loaded.

50μg of protein from whole cell extracts of yeast transformed with pYEDP10-1/+mGST and pYEDP10-1/-mGST were loaded.

6.3.7 Expression of the rat microsomal glutathione S-transferase as a fusion protein with the *Schistosoma japonicum* glutathione S-transferase.

6.3.7a Introduction.

The production of fusion proteins as a means of achieving successful expression of foreign proteins in bacteria has long been employed (reviewed by Uhlen and Monks 1990). The rationale behind this strategy is several fold. The fusion of foreign peptides to host proteins is thought to increase stability and avoid proteolysis, which has been demonstrated for a number of small proteins (Marston 1986). A number of protein properties such as hydrophobicity (Marston 1986) and high proline content (Schien 1989) have been implicated in causing inclusion body formation, as demonstrated by expressing different regions of the protein and observing different solubilities. Removal of the hydrophobic domain of the Moloney murine leukemia virus (MMLV) allowed expression in a soluble form, which was not possible with the wild type protein (Kotewicz *et al* 1985). The expression of insoluble proteins as fusions may act to"dilute" out these unfavourable characteristics and thereby increase solubility. The fusion of proteins with the highly stable and soluble *Schistosoma japonicum* GST has been implicated in the increase in solubility of hydrophobic proteins (Smith and Johnson 1988).

Only the T7 promoter could drive the expression of the rat microsomal GST protein, but the enzyme was in an insoluble form. Other less powerful promoters were only able to generate mRNA. A possible explanation was proposed that the rat microsomal GST is unstable in the bacterial environment, and coupled with its hydrophobic nature, will only exist in an insoluble form. A translational block was also suggested, but a range of promoters that added a different N-terminal sequence to the protein did not alleviate the problem. In Section 6.3.5 the problem of purification of the protein was discussed and one of the major advantages of fusion proteins is that the carrier protein is often able to be affinity purified, which facilitates a simple single step purification. After purification, the carrier protein is generally removed by use of an specifically engineered proteolytic site.

Fusion of the microsomal GST to a cytosolic GST, *Schistosoma japonicum,* on initial inspection be might not seem an obvious choice due to potential problems in separating the two GSTs after proteolytic cleavage. However, the distinct properties of the microsomal GST and cytosolic GSTs have been extensively discussed throughout this thesis and is reflected in the different purification schemes. In this respect the microsomal GST has been reported not to bind to glutathione or S-hexyl affinity columns (Morgenstern and DePierre 1985), which have been used to purify the cytosolic GSTs including the *Schistosoma japonicum* GST (Smith and Johnson 1988). It was hoped that once purified the microsomal GST would be stable in a detergent environment or reconstituted in a lipid environment.

6.3.7b Expression of the *Schistosoma japonicum* glutathione S-transferase fusion proteins.

Figure 6.17 shows the pGEX expression vector into which a full length and truncated (at Lysine 41) PCR derived cDNA were cloned at the BamH1 site. Figure 5.17a shows a coomassie blue stained SDS-PAGE gel of whole cell, cell debris, 18000g supernatant and pellet samples from bacteria transformed with pGEX 2-T, and the two expression constructs. The *Schistosoma japonicum* GST is 26KDa (Smith *et al* 1986) and when expressed in this system it is located predominantly in the supernatant. The two fusion proteins are located predominantly in the 18000g pellet, however Western blot analysis, using the rat microsomal GST antibody, revealed the presence of some soluble protein in the supernatant (Figure 6.18b). Interestingly, the amount of soluble protein was larger in the truncated microsomal GST fusion protein, which supports the idea that the hydrophobic nature of the protein is responsible for its insolubility in bacteria.

The CDNB activity of the *Schistosoma japonicum* GST is easily detectable and a convenient measure of active protein present in the various samples, however the contribution made by the microsomal GST in this situation would probably be negligible. From Figure 6.18a the amount of *Schistosoma japonicum* GST protein is approximately equivalent to the amount of fusion proteins observed in the whole cell. However, the CDNB activity of the fusion proteins was reduced to a tenth of the wild type

Figure 6.17 pGEX-2T expression vector.

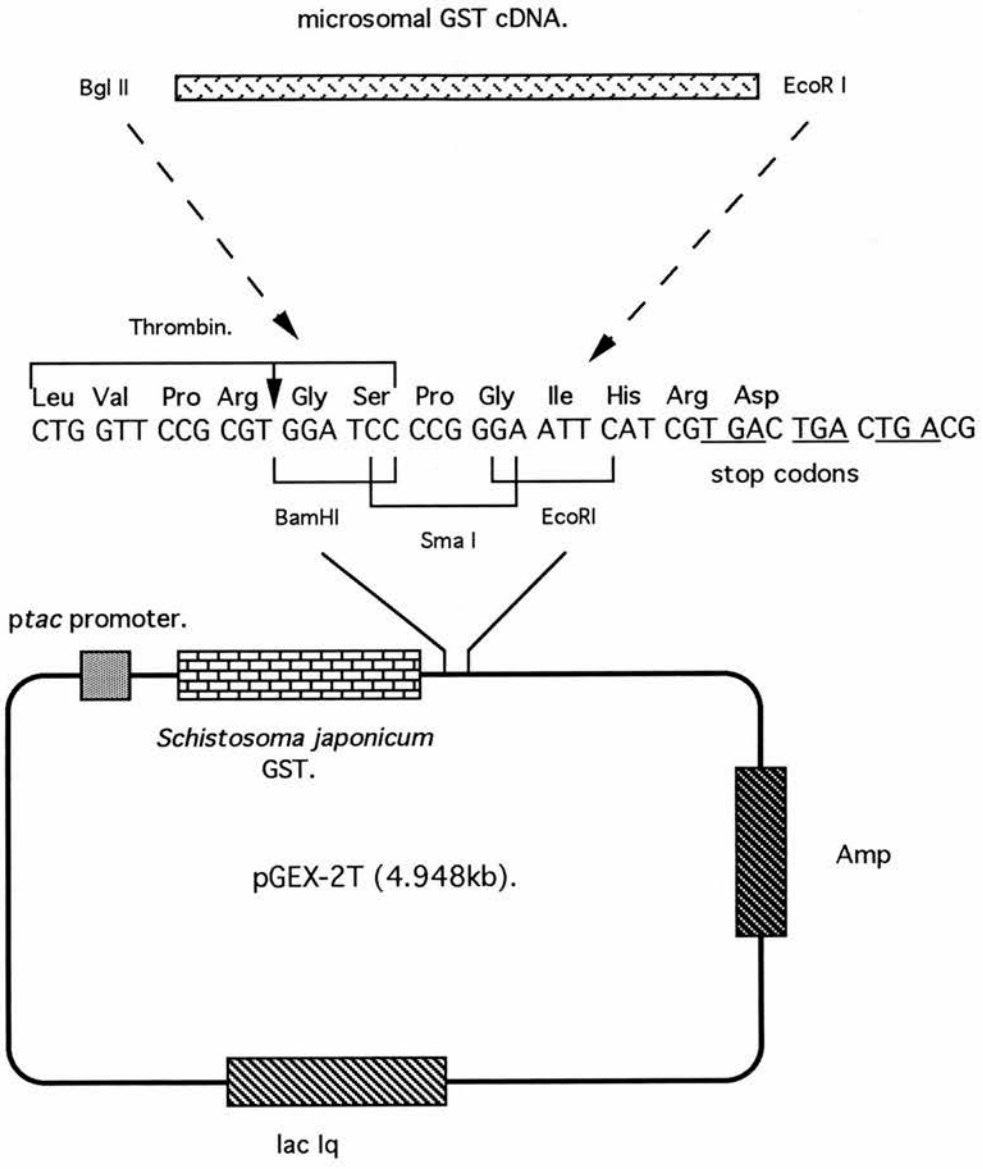
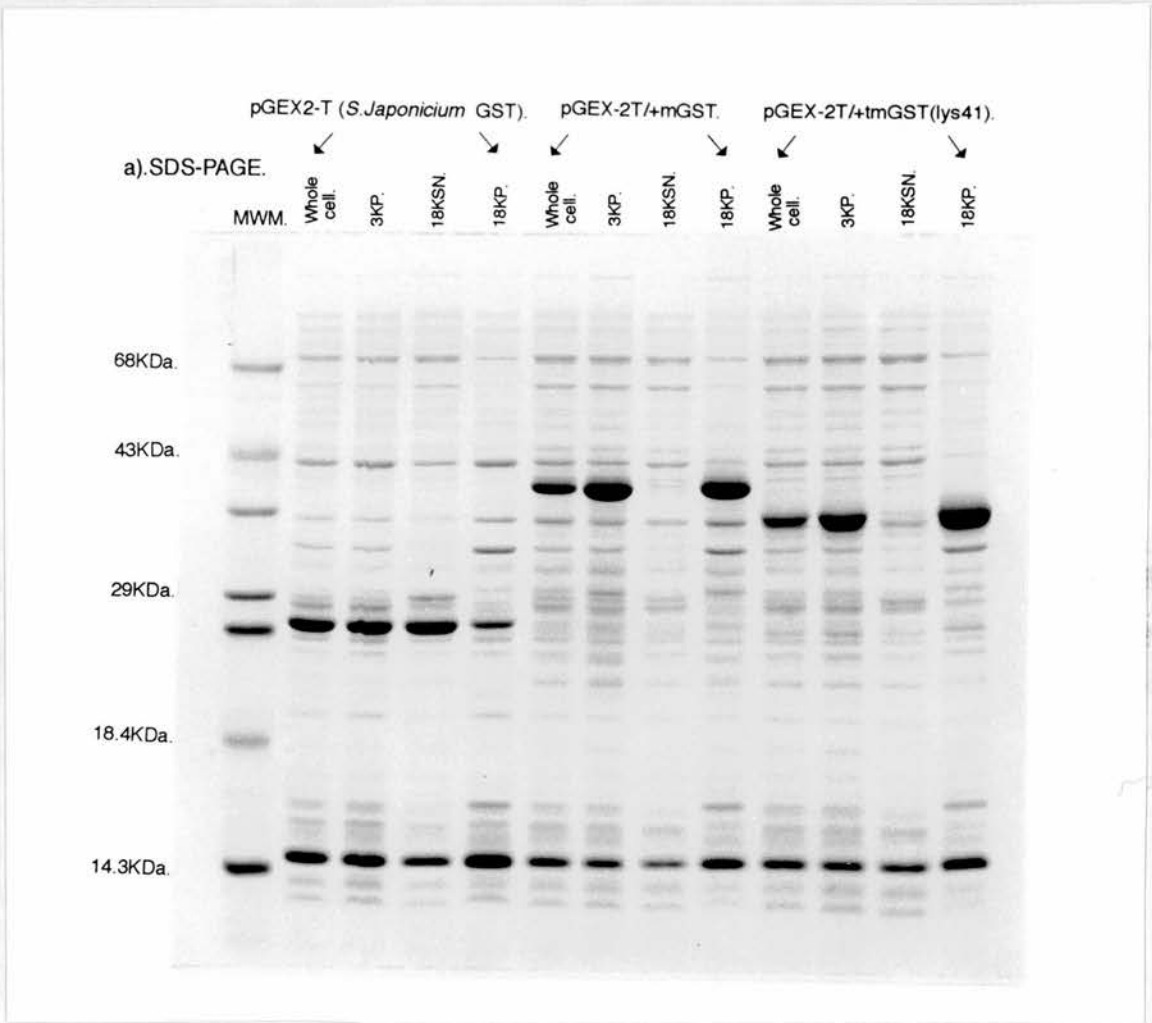


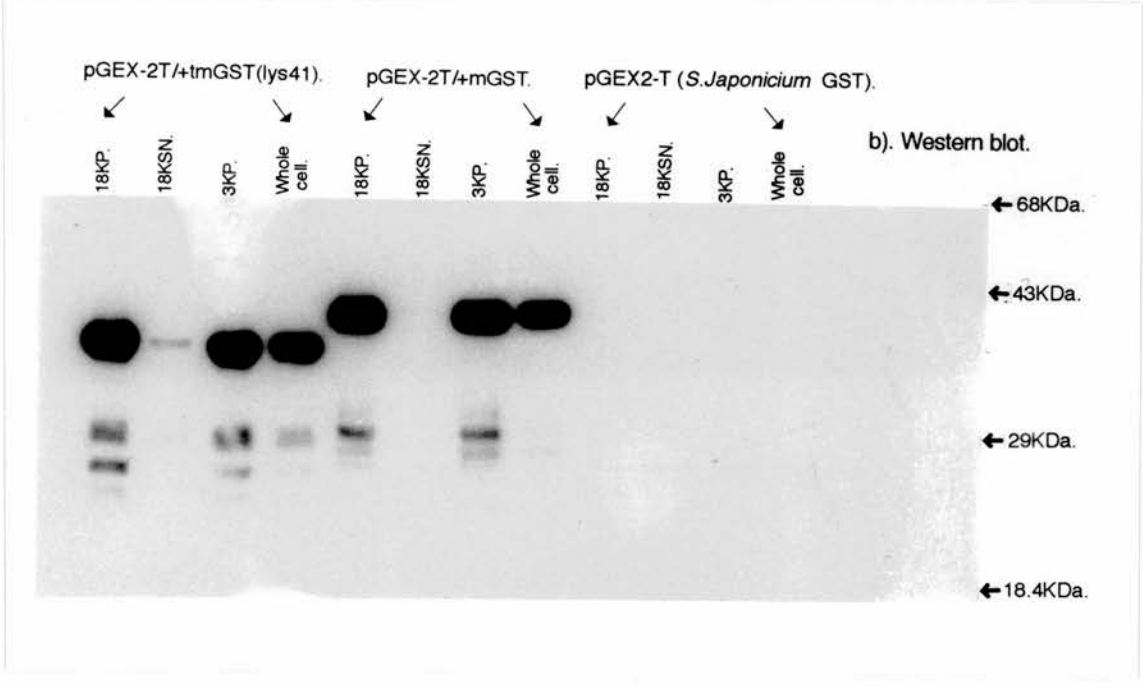
Figure 6.18. Expression pGEX2-T (parental vector) and pGEX2-T/microsomal glutathione S-transferase fusion proteins in bacteria [a). SDS-PAGE].



30μg of total protein loaded.

MWM: molecular weight markers.

Figure 6.18. Expression pGEX2-T (parental vector) and pGEX2-T/microsomal glutathione S-transferase fusion proteins in bacteria [b). Western blot analysis].



10μg of total protein loaded.

NB. the Western blot is the mirror image of the SDS-PAGE.

Schistosoma japonicum GST value in the whole cell, which indicates that~90% of the fusion protein is insoluble and this confirms the observations made with the Western blot. The CDNB activity found in the 18000g pellet was larger in the fusion protein samples, which suggested that some protein may be loosely associated with the membranes or the inclusion bodies in an active form and this could be easily removed by washing in a Triton X-100 containing buffer.

6.3.7c Purification of the *Schistosoma japonicum* glutathione S-transferase fusion proteins.

The fusion proteins were purified by the method described by Smith and Johnson (1988) with the addition of 0.1% Triton X-100 to buffers in order to reduce bacterial protein contamination and to provide a favourable environment for the microsomal GST. The CDNB activity of the *Schistosoma japonicum* GST and the use of a UV detector allowed the purification of the fusion proteins to be followed. The CDNB elution profiles of the two fusion proteins from the glutathione-Agarose affinity columns are shown in Figure 6.19. The coomassie stained SDS-PAGE and the western blot analysis, using the rat microsomal GST antibody, for the purification of the two fusion proteins is shown in Figure 6.20 (a,b,c and d). As can be seen from the SDS-PAGE (Figure 6.20 a and b) both fusion proteins eluted as two major bands, one corresponding to the molecular weight of fusion protein and the other slightly greater than 26KDa, the molecular weight of the *Schistosoma japonicum* GST. There is also a high molecular weight bacterial contaminant that appears in both purifications. The western blot analysis (Figure c and d), using the rat microsomal GST antibody, demonstrated the two lower molecular weight bands to contain rat microsomal GST sequences with a number of intermediary cross-reacting bands between them. These results suggest that the microsomal GST portion of the fusion proteins is susceptible to proteolysis, when in the soluble form in the bacteria. This concurs with previous suggestions that the failure to express microsomal GST protein in bacteria was due to proteolysis of the protein. In the Western blot of the full-length microsomal GST fusion protein (Figure 6.20 c) there is a third cross-reacting band close to the

Figure 6.19a Elution of pGEX2T/+mGST from GSH-agarose.

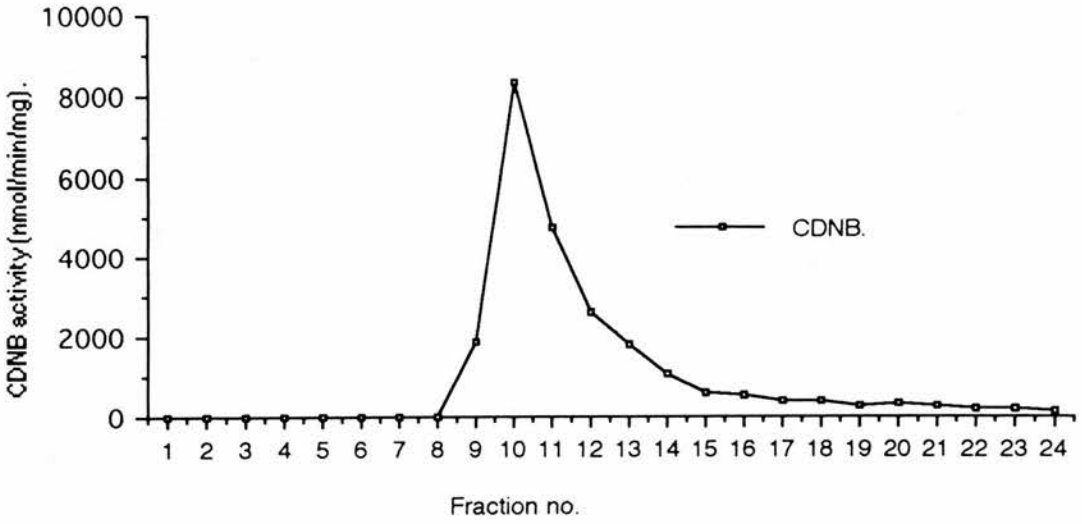


Figure 6.19b Elution of pGEX2-T/+tmGST(lys41) from GSH-agarose.

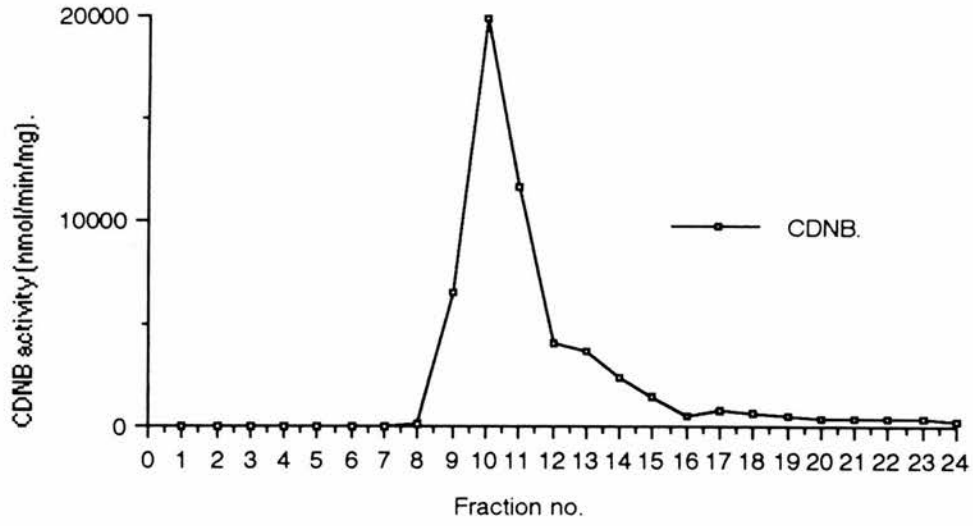
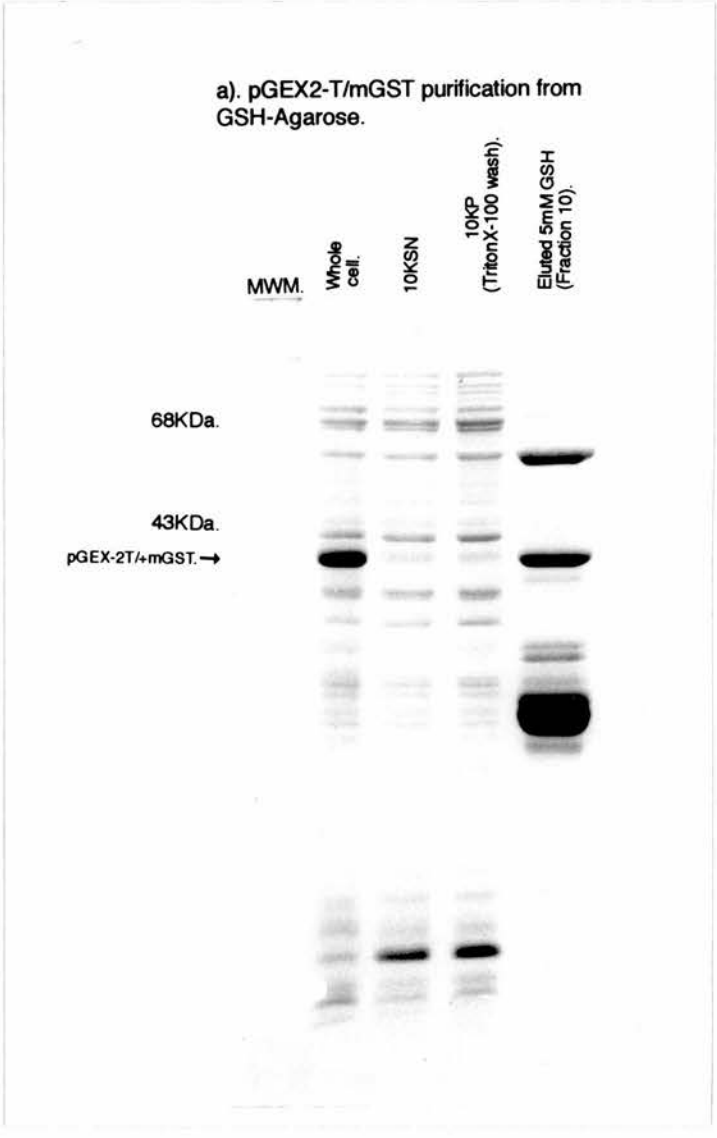


Figure 6.20. Purification of pGEX2-T/microsomal glutathione S-transferase from bacteria (SDS-PAGE).

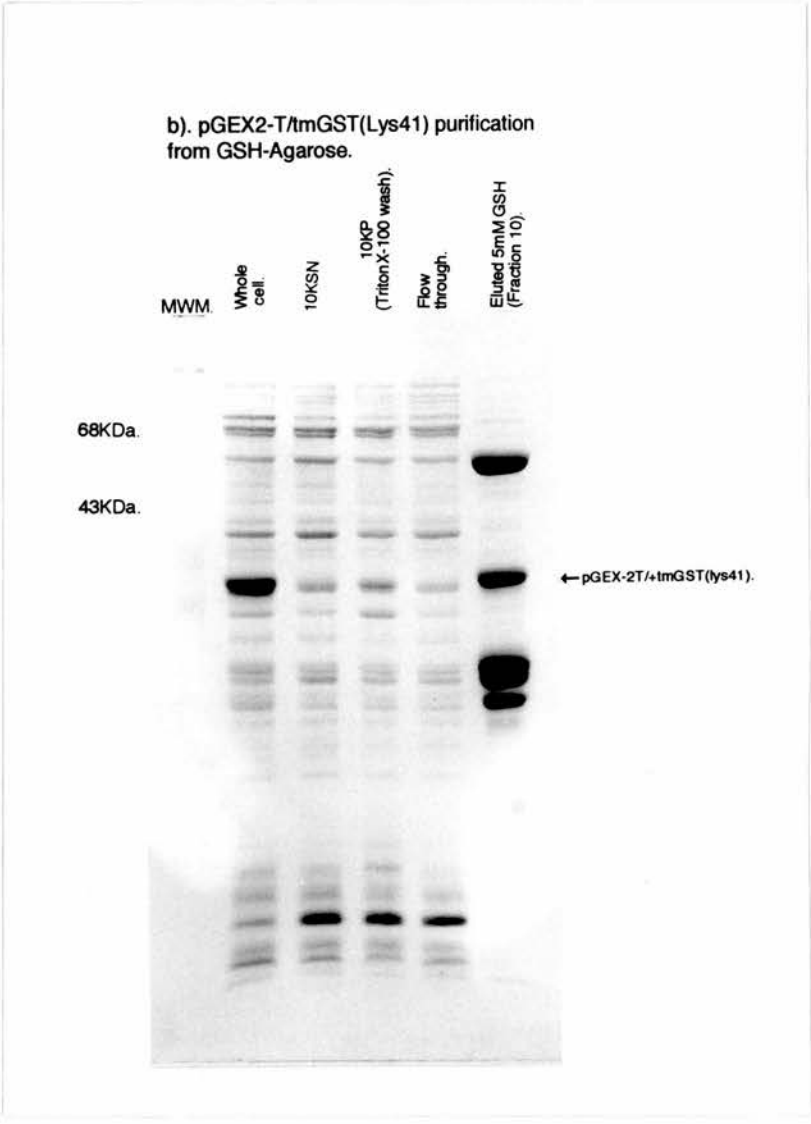
a) pGEX2-T/mGST.



MWM: molecular weight markers.

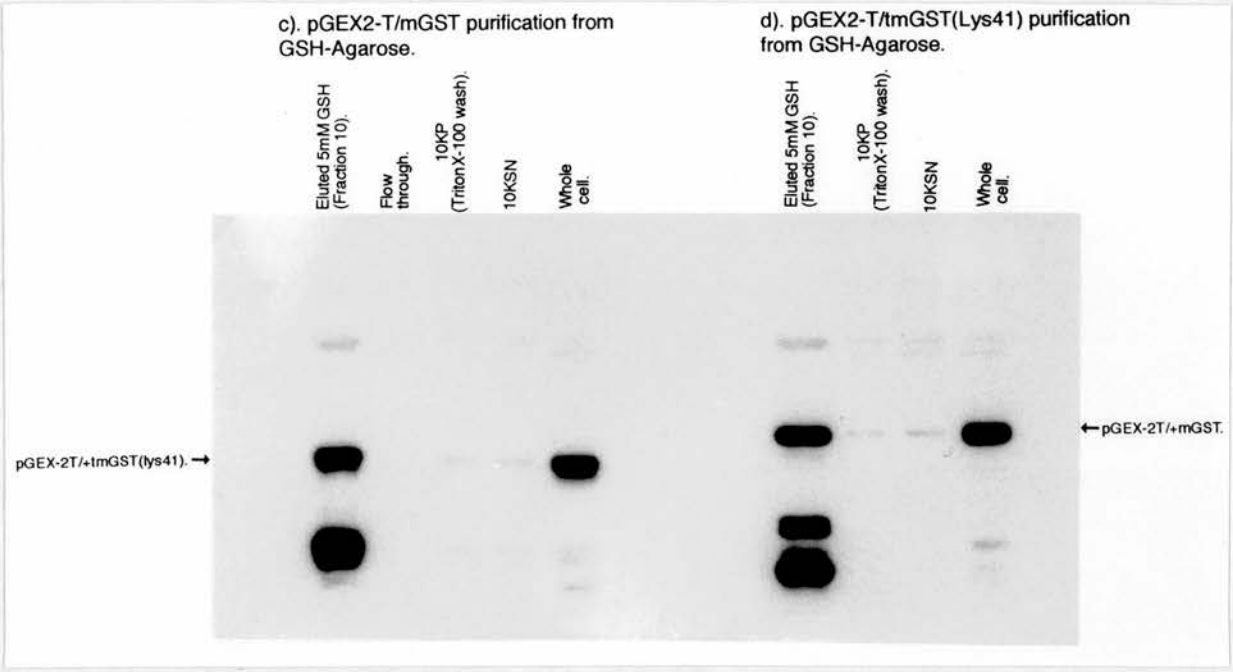
30μg of total protein loaded.

Figure 6.20. Purification of pGEX2-T/microsomal glutathione S-transferase from bacteria (SDS-PAGE).
b) pGEX2-T/tmGST(Lys41).



MWM: molecular weight markers.
30μg of total protein loaded.

Figure 6.20. Purification of pGEX2-T/microsomal glutathione S-transferase from bacteria (Western blot).



10μg of total protein loaded.

NB. Western blot is the mirror image of the respective SDS-PAGE.

~26KDa species. This could be derived from proteolytic activity at the lysine 41 region which has been shown to be a protease-sensitive site in the native protein (Morgenstern *et al* 1989 and Lundqvist and Morgenstern 1992a). Further evidence to support this idea comes from the absence of this band in the Western blot analysis of the truncated microsomal GST fusion protein, which was derived from this region of the protein (Figure 6.20 d). The fact that the ~26KDa species cross reacts with the microsomal GST antibody and is slightly larger than 26KDa suggests that some microsomal GST sequences are still attached. No fragments smaller than 26KDa were observed suggesting they must have been rapidly turned over in the bacterial cell.

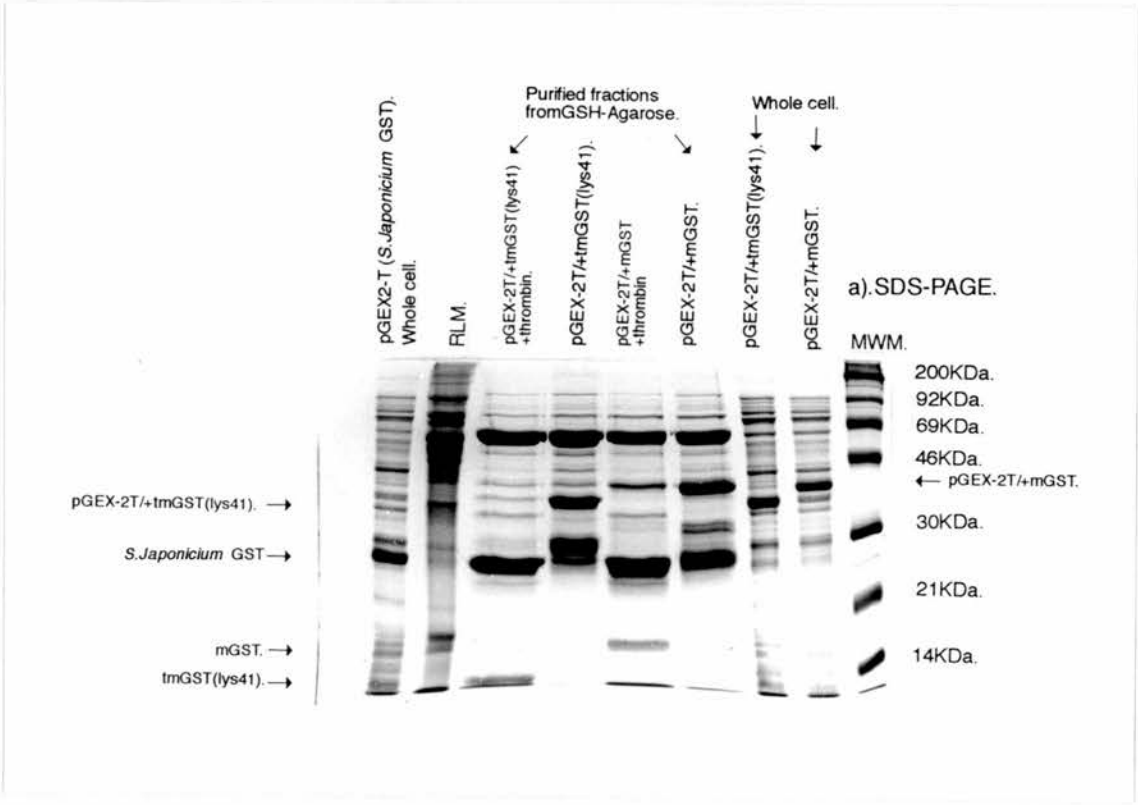
6.3.7d Removal of the *Schistosoma japonicum* glutathione S-transferase by thrombin proteolytic cleavage.

The pGEX-2T vector contains a thrombin cleavage site immediately upstream of the multiple cloning site (Figure 6.17). Figure 6.21 a and b show the coomassie blue stained SDS-PAGE and the equivalent western blot, probed with the rat microsomal GST antibody, of both fusion proteins before and after cleavage with thrombin, respectively. As can be seen, thrombin treatment releases a 17KDa and 12KDa fragment from the full-length and truncated microsomal GST fusion proteins, respectively, and both these peptides cross react with the antibody. A 26KDa protein is also released and does not cross react with the rat microsomal GST antibody, this is the *Schistosoma japonicum* GST and it is slightly lower than the cross reacting band of similar molecular weight observed before cleavage. With both fusion proteins a small amount of protein remains resistant to cleavage. However noticeably more is cleaved in the truncated microsomal GST fusion protein, which indicates the sensitivity of the protein to proteases in this region.

6.3.7e Separation of the *Schistosoma japonicum* and microsomal glutathione S-transferases after proteolytic cleavage.

Initially attempts were made to reabsorb the fusion proteins back onto

Figure 6.21. Cleavage of pGEX2-T/microsomal glutathione S-transferase with thrombin (SDS-PAGE).



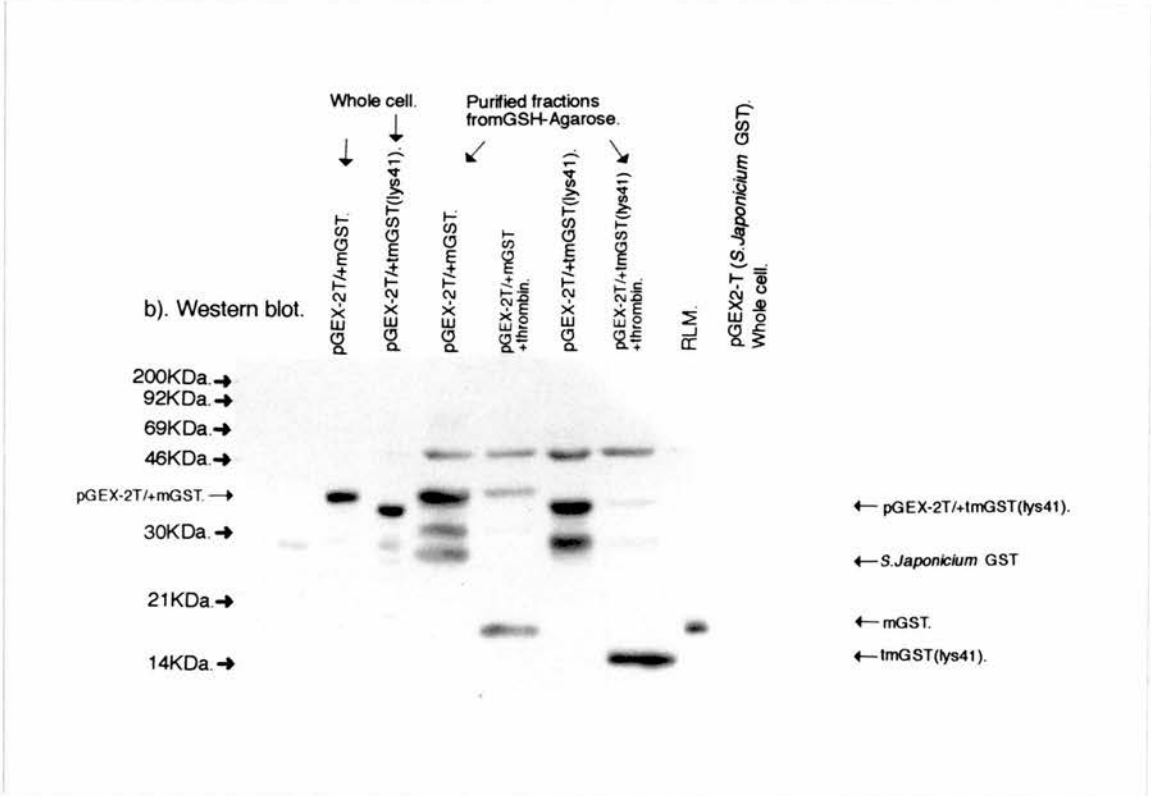
MWM: molecular weight markers.

30μl of the cleavage reaction, before and after the addition of thrombin, was loaded.

30μg of protein from bacterial whole cell extracts were loaded.

30μg of rat microsomal protein loaded.

Figure 6.21. Cleavage of pGEX2-T/microsomal glutathione S-transferase with thrombin (Western blot).



30μl of the cleavage reaction, before and after the addition of thrombin, was loaded.

10μg of protein from bacterial whole cell extracts were loaded.

10μg of rat microsomal protein loaded.

the GSH-Agarose after purification and to cleave the microsomal GSTs while the *Schistosoma japonicum* GST was still attached to the GSH-Agarose. However neither the full-length nor the truncated microsomal GST were released. Not only was the efficiency of the cleavage greatly reduced, but the cleaved fragments would not dissociate from the matrix. This was judged to be partially due to non-specific interactions (probably hydropohpbic despite the presence of Triton X-100) and partly due to an affinity for the GSH ligand. These assumptions were made on the basis that GSH could remove some protein, but the majority was removed by boiling the agarose beads in SDS-PAGE sample buffer. However neither of these methods would allow the separation of the two GSTs.

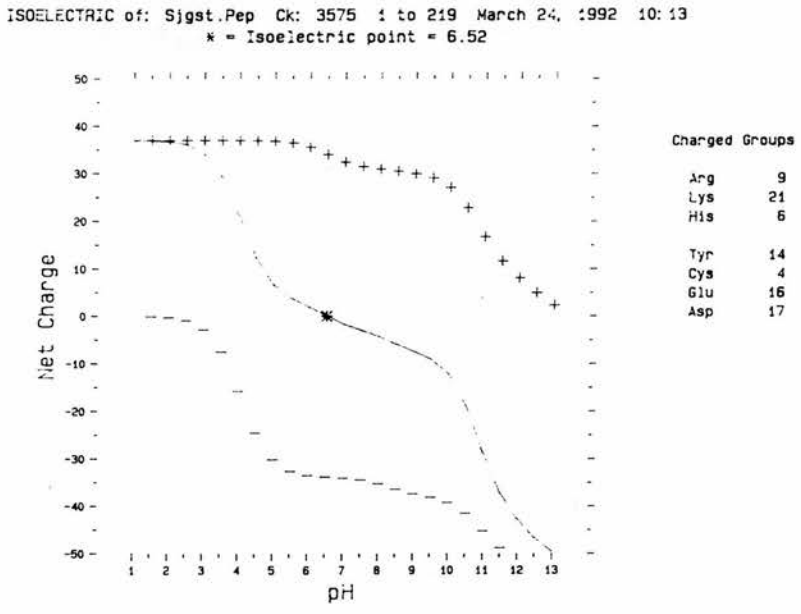
The microsomal GST is an extremely basic protein with a pI of 10.1 (Morgenstern *et al* 1983), whereas the *Schistosoma japonicum* GST is most closely related to the rat Yb_1, a μ class enzymes with near neutral pI (Smith *et al* 1986). The Wisconsin programme can be used to calculate the PI of a protein on the basis of its amino acid sequence and was duely used to calculate the pI of the *Schistosoma japonicum* GST, the microsomal GST and the fusion of the two, the results of which are shown in Figure 6.22 a b and c, respectively. Based on this data it was possible to design an ion-exchange FPLC strategy to separate the three different polypeptides due to their markedly different pIs. At pH 9.0, it was hoped that the microsomal GST would be positively charged and not stick to the cationic Mono Q matrix. The other peptide species being negatively charged at this pH would adhere and would later be eluted with sodium chloride (NaCl).

Figure 6.23 shows the elution profile of the FPLC column loaded with a sample of full-length microsomal GST fusion protein, which had previously cleaved with thrombin. Three major protein peaks were observed, the first in the flow-through, fractions 3-5; the second during the first part of the 0.5M NaCl gradient, fractions 33-45; and the third peak at 100% 0.5M NaCl, fractions 52-55. However, only the first two contained any CDNB activity and the third peak was shown to be the high-weight bacterial contaminant of approximately 69KDa (seen in Figure 6.20) by SDS-PAGE analysis.

The silver stained SDS-PAGE in Figure 6.24 of the fractions in the two peaks from the FPLC that contained the CDNB activity. Although it was speculated that the microsomal GST would pass through the column in the

Figure 6.22. The computer calculation of isoelectric points for the *Schistosoma japonicum* glutathione S-transferase, rat microsomal glutathione S-transferase and the pGEX2-T/microsomal GST fusion protein.

a). pI of the *Schistosoma japonicum* GST.



b). pI of the rat microsomal GST.

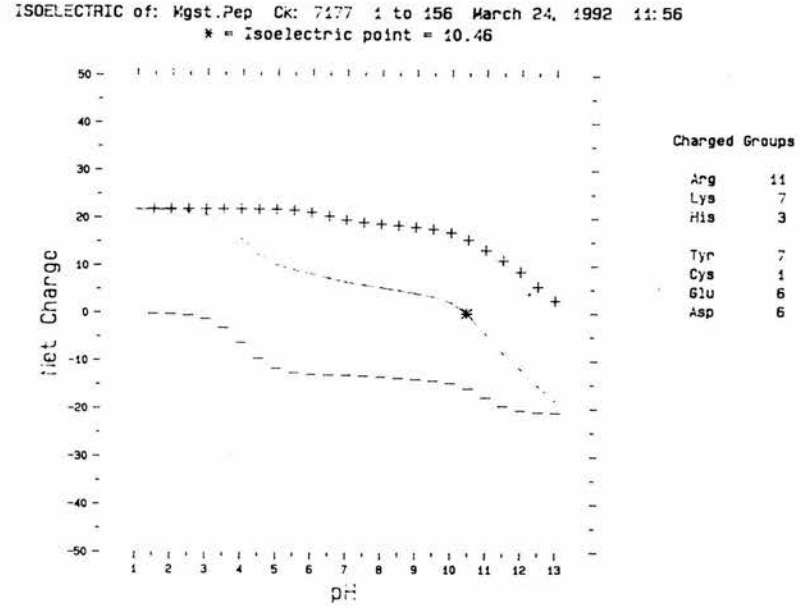


Figure 6.22. The computer calculation of isoelectric points for the *Schistosoma japonicum* glutathione S-transferase, rat microsomal glutathione S-transferase and the pGEX2-T/microsomal GST fusion protein (continued).

c). pI of the pGEX2-T/microsomal GST fusion protein.

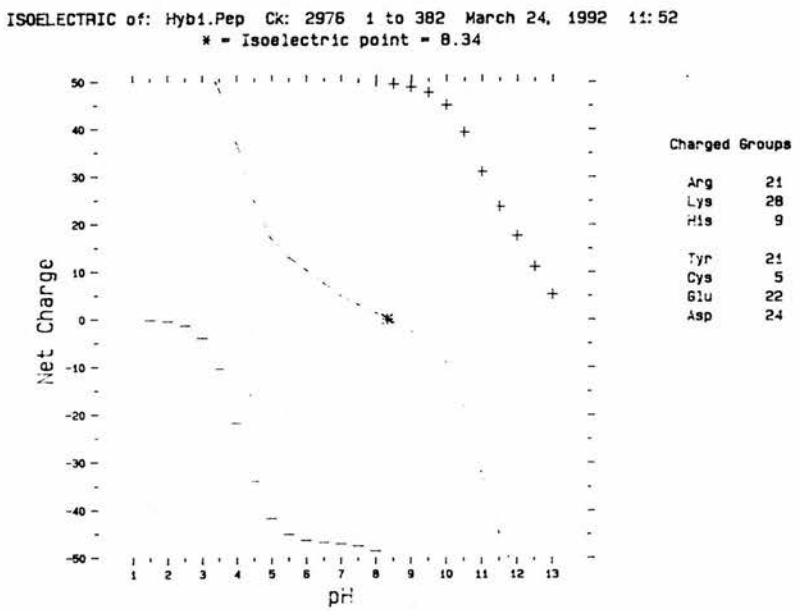


Figure 6.23 Elution profile from Mono Q column (FPLC).

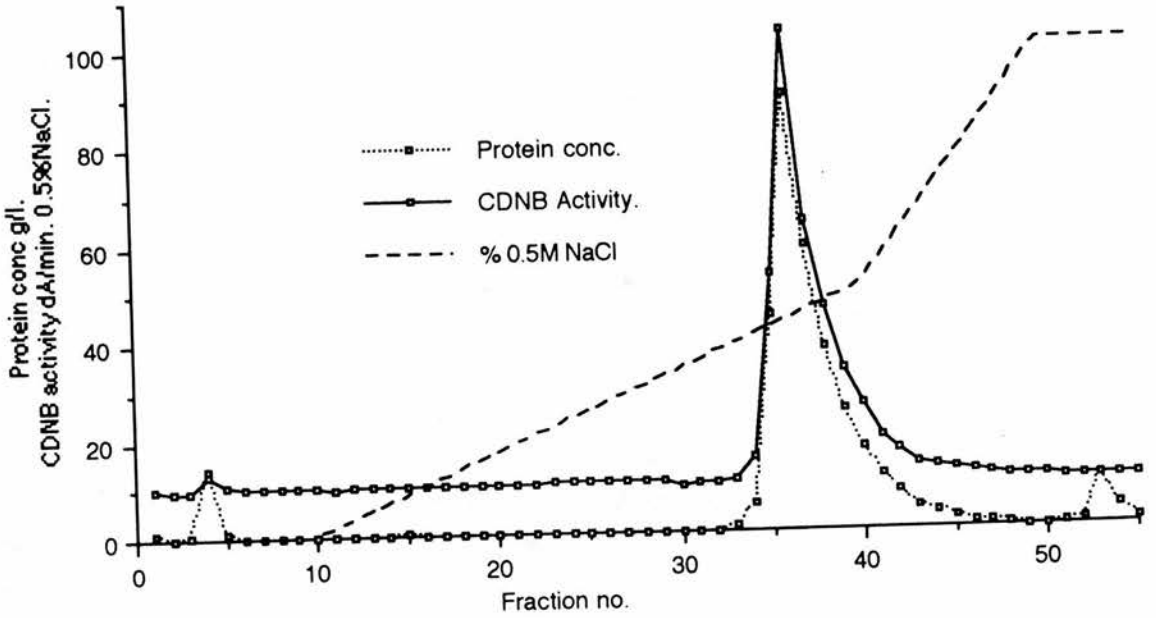
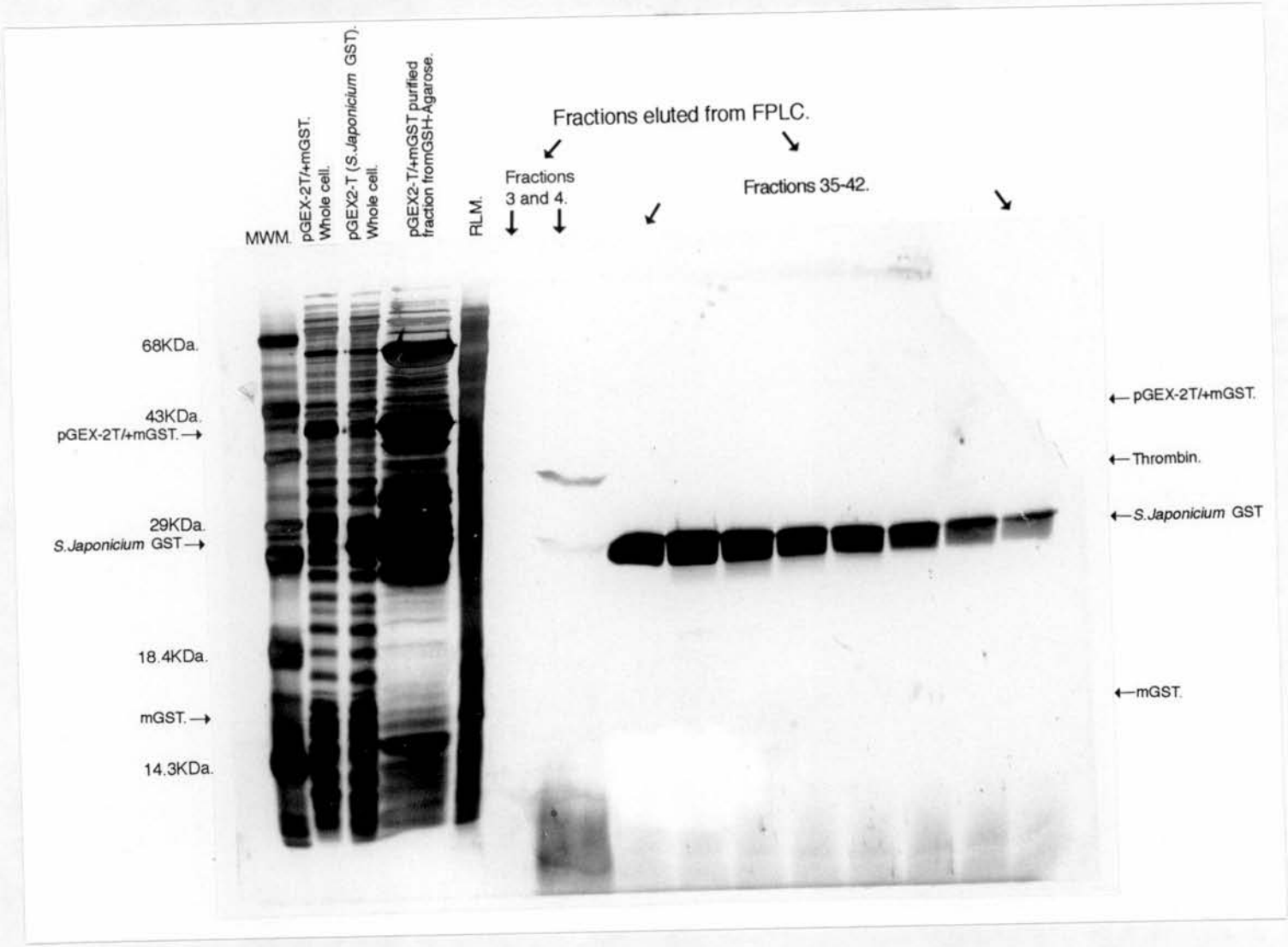


Figure 6.24. Silver stained gel of the first two protein peaks eluted from the Mono Q FPLC.



MWM: molecular weight markers.

RLM: 10μg of rat microsomal protein loaded.

100μl of fraction from the FPLC loaded.

10μg of total protein from all other samples loaded.

flow-through, the activity in this peak was due to *Schistosoma japonicum* GST, which was present with thrombin. These two proteins may have associated in some way during the proteolytic process and hence altered the interaction of the *Schistosoma japonicum* GST with the column. The second peak contained the bulk of the *Schistosoma japonicum* GST protein which eluted at the predicted point indicating that the FPLC was operating properly. Unfortunately no microsomal GST could be recovered in any fractions. It is possible that once again the hydrophobic nature of the protein caused it to interact with the matrix of the column. The purification procedure of the native protein uses CM-Sepharose cation exchange and the protein can be recovered (Morgenstern *et al* 1982). The native protein is purified as a trimer (Morgenstern *et al* 1982), and it is unclear how the subunits of the microsomal GST would associate when cleaved from the *Schistosoma japonicum* GST. However, the cytosolic GSTs must be in a dimer in order to be active (Aceto *et al* 1992). In Section 6.3.5 the recombinant protein purified from a denatured state appeared to adhere to CM-Sepharose. It may be that if not correctly folded, hydrophobic residues normally found in the interior of the protein are exposed, making the protein more "sticky".

To resolve these proteins the concentration of detergent in the buffers could be raised to limit hydrophobic interactions with the column matrix. Alternatively, a hydrophobic FPLC column could be used to remove the microsomal GST from the other more hydrophylic species.

The lack of signal sequence in the microsomal GST has allowed a cotranslational process of insertion into the membrane to be proposed (DeJong *et al* 1988). Whether the formation of a trimer is part of this process, or requires other factors such as molecular chaperones (Nilsson and Anderson 1991). Alternatively, the trimer maybe achieved non enzymatically, due to it been energetically favourable. The native rat microsomal GST purified from liver has been successfully reconstituted into phosphatidyl choline liposomes (Andersson and Morgenstern 1992). It is not possible to predict the oligomeric make up of the microsomal GST, produced by this recombinant technology, in a reconstituted system. However, there is a report in the literature describing the successful reconstitution of separately prepared α and $\beta\gamma$ subunits of the G protein $G_{q/11}$ and the recombinant m1 muscarinic receptor (Berntein *et al* 1992), but of course this is a heteromeric

structure.

6.4 Summary and future work.

This Chapter has described the generation of an *E.coli* expression system for the microsomal GST. The failure to express the microsomal GST in bacteria, as judged by these experiments, is probably due to the highly hydrophobic nature of the protein and a high susceptibility to bacterial proteolysis.

The expression of the microsomal GST under the control of the T7 promoter yielded insoluble protein, which on recovery from the inclusion bodies was inactive. The use of different solubilising agents was discussed. pET vectors are now available with a facility to clone a string of histidines at the C terminus of the protein, this allows the expressed protein to be purified under denaturing conditions using a nickel affinity column. However although this may increase the yield of denatured protein from the denatured state, it does not help the refolding of the protein. Careful experiments would need to be carried out to determine the exact conditions to achieve correct folding for the microsomal GST. Such conditions depend on the nature of the individual protein and are not considered to be empirical (Mozhaev *et al* 1988, Kohno *et al* 1990).

The fusion system produced soluble protein that could be efficiently purified and cleaved from the carrier protein. However the problem lay in resolving the two GSTs. However, this could be overcome by fusing the microsomal GST to a different protein. A series of protein A fusion vectors (gift from Dr. A.Boyd), the pAX vectors were obtained, and it was possible to clone in a PCR-generated microsomal GST cDNA containing a thrombin site 5' of the N-terminus. The resulting protein could be affinity purified using IgG sepharose, and the protein was released using 0.5M acetic acid pH 3.4 followed by dialysis. After freeze-drying the protein was rather insoluble even in a Triton X-100 containing buffer, which suggested the protein had aggregated or folded improperly. CDNB activity could not be measured either after freeze-drying or at any point in the process, such as on the IgG sepharose.

A suitable protein was required for fusion with the microsomal GST

that was hydrophylic, affinity purifiable in non-denaturing conditions and did not have CDNB activity. The bacterial phosphofructokinase A (PFKA) was available in a pEMBL 8 expression vector (pHL1) under the control of *lac* promoter (Lau *et al* 1987). The cDNA encodes a 322 amino acid protein and at nucleotide position 814bp (relative to the ATG) there is a *Cla* 1 site. This site is 150bp from the stop codon and encodes the last 50 amino acids of the protein. If the protein was to be cleaved at this point the following C terminal secondary structures would be lost; the C-terminal region of α helix 11; β sheets J and K; and α helix 12 (Hellinga and Evans 1985). From the crystal structure some of the residues in the ADP effector site located in α helix 13 would be lost (Schrimer and Evans 1990), in fact part of α helix 13 can be removed by limited proteolytic cleavage with subtilisin, which results in abolition of effector properties but retains catalytic properties (Le Bras and Garel 1982).

From the microsomal GST cDNA derived for the pAX vector it was possible to create a microsomal fusion protein with PFKA containing a thrombin cleavage site, by cloning into the *Cla* 1 site. For the manipulations involving the *Cla* 1 site the plasmids had to be passed through a dam$^-$ strain of *E.coli*, JM101 dam$^-$, to ensure the DNA was unmethylated. The PCR oligo used to generated the microsomal GST cDNA for directionally cloning into the pAX vector had generated *Eco*R1/*Bam*H1 fragment encompassing the microsomal GST cDNA and a N terminal thrombin site. This cDNA was removed by the EcoR1 site, end filled then *Bam*H1 *cleaved*. This fragment was cloned into puc 18 in a double ligation with the *Bam*H1/*Cla*1 fragment of PFKA removed from pHL1 (pEMBL 8 containing the *pfk A* gene in a *Bam*H1 fragment, Lau *et al* 1987). Wild type pEMBL 8 was generated by the removal of the *pfk A* gene by a *Bam*H1 digest followed by CIAP treatment. The *Bam*H1 fragment containing the PFKA and microsomal GST fusion was excised and cloned into the phosphatased pEMBL 8. The microsomal GST contains a unique *Sst* 1 site 375 bp from the TAA, while there is a unique *Hind* III site 3' of the *Bam* H1 site in pEMBL8. The orientation of the *Bam* H1 fragment was assessed by a double digest using *Sst* 1 and *Hind* III, fragments of the correct orientation released a insert size of approximately 400bp, while the reverse orientation gave 1kb fragment.

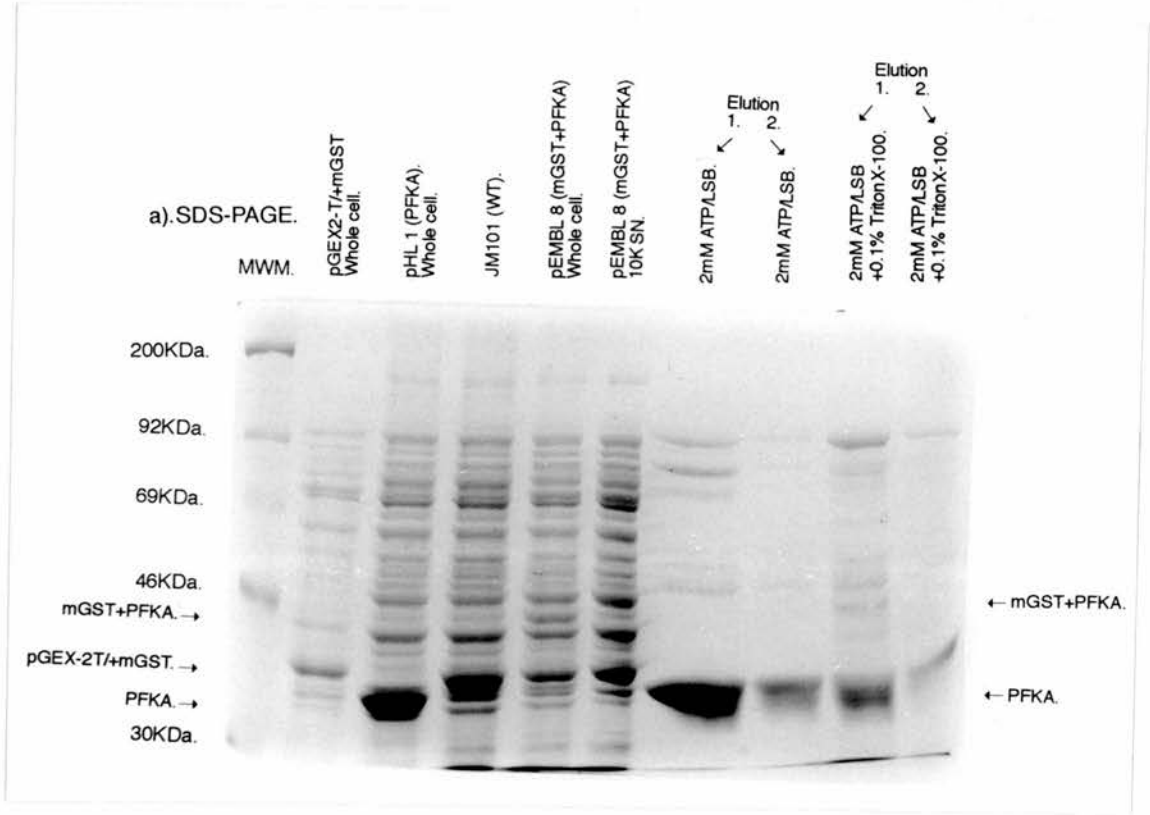
Figure 6.25 shows the coomassie blue stained SDS-PAGE and western blot analysis, using the rat microsomal GST antibody, from the whole cell and supernatant of bacteria transfected with this expression construct and the pEMBL 8. Although the amount of microsomal GST PFKA fusion product is lower than the wild type PFKA protein, as judged on the SDS-PAGE (Figure 5.24a), there is some soluble product as seen in the western blot analysis (Figure 5.24b).

A batch purification was preformed using Cibacron blue 3GA sepharose and the method modified from a column purification described by Lau *et al* (1987). As can be seen from Figure 5.24b in the presence of Triton X-100, the fusion protein could be purified and released from the affinity matrix by ATP.

Although the strategy essentially worked the system needed some modifications. Firstly, large quantities of wild type PFKA were been purified by this procedure, but this could easily be overcome by using the DF1020 strain of bacteria from which the *pfkA* gene had been deleted (Daldal *et al* 1983). Secondly, cibacron blue 3GA is a non-specific ligand which is recognised by the binding site of nucleotide dependent enzymes, but it also interacts with hydrophobic proteins and has been shown to be an inhibitor of the microsomal GST (Mosialou and Morgenstern 1990). This could potentially impair recovery of the protein from the affinity matrix, although other more specific affinity matrices are available for the purification of ADP/ATP dependent enzymes, such as 5' AMP-Sepharose 4B (Pharmacia 1983).

Even if a recombinant microsomal GST could be generated in this manner there is no guarantee that the active quaternary structure of the protein would be achieved. However if active protein was generated the experiments to investigate the catalytic and activation processes would certainly yield some interesting findings.

Figure 6.25. Expression and purification of the phosphofructokinase A/microsomal glutathione S-transferase fusion proteins in bacteria (SDS-PAGE).



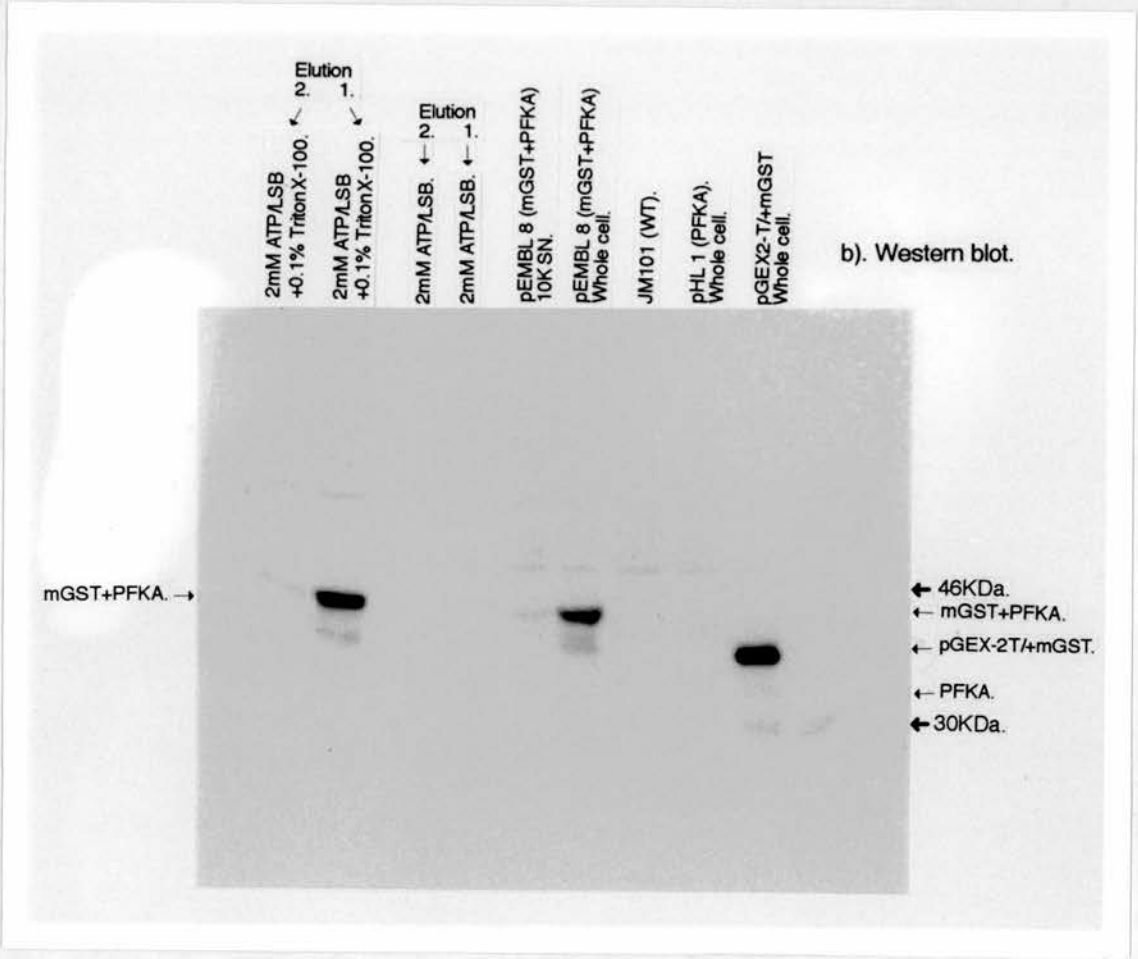
MWM: molecular weight markers.

100µl of fraction from the reactive blue sepharose was loaded.

10µg of total protein from all other samples loaded.

pGEX2-T/+mGST was used as a standard.

Figure 6.25. Expression and purification of the phosphofructokinase A/microsomal glutathione S-transferase fusion proteins in bacteria (Western blot).



100µl of fraction from the reactive blue sepharose was loaded.

30µg of total protein from all other samples loaded.

pGEX2-T/+mGST was used as a standard.

NB. Western blot is a mirror image of SDS-PAGE.

Chapter 7: Discussion and future work.

The microsomal glutathione S-transferase possesses a fascinating regulatory mechanism that is only just beginning to be understood in relation to the physiological changes that occur within the cell. It would appear that the various forms of cellular insult could potentially increase the activity of the enzyme such as disruption of thiol status resulting in a rise in GSSG. Alternatively, reactive metabolites or free radicals, which mediate lipid peroxidation events, can also bring about activation of the enzyme. In most cases the enzyme activating species are thought to bring about an increment in activity by covalent modification of cysteine 49. Although there is some debate as to the exact nature of the covalent modification, and indeed the activating species, enzyme kinetic studies have revealed a confirmational change occurs in the active (G) site. This allows the enzyme to utilise the binding energy of glutathione more efficiently than in the unactivated form. In addition, the kinetic parameters move closer towards the cytosolic GSTs. However, the amino acid sequence data available shows no appreciable sequence homology between the microsomal and cytosolic forms, which perhaps suggests not only a different catalytic mechanism but a subtly different function.

The endogenous regulation of the rat and mouse microsomal glutathione S-transferase.

The cytosolic GSTs are subjected to to tissue-specific and sex-specific regulation (Niems and Hales 1979; Lamartiniere 1981; Hayes and Mantle 1986; Hatayama *et al* 1986; McLellan and Hayes 1987). In addition hormonal control and xenobiotcs can regulate the expression of these proteins. The microsomal GST differs from the cytosolic GSTs in that the transcription of the gene can not be greatly affected by xenobiotics (Morgenstern *et al* 1980; DeJong *et al* 1988; Morgenstern *et al* 1990). However studies investigating the endogenous regulation of the mouse liver microsomal GST, showed that the protein was developmentally regulated in the liver, lung, kidney and testis, and these

results implied the involvement of the pituitary gland. Further studies directly demonstrated that the pituitary gland was involved in regulating the expression of this protein in the mouse liver. The experimental approach was to surgically remove the pituitary gland nine weeks after birth, and animals treated in such a manner showed depressed levels of microsomal GST expression compared with the controls. The other experimental rodent used was the rat, but the results suggested that the pituitary gland did not regulate expression of the microsomal GST in this species.

A slightly higher level of expression of the liver microsomal GST was observed in male control mice than female mice, however the difference was not as marked as the sexual dimorphism observed in the expression of the Yf GST subunit in this species (Hatayama *et al* 1986). Treatment of mice with dexamethasone caused a slight induction in the male of microsomal GST protein expression. Dexamethasone, a synthetic glucacorticoid, has been shown to interfere with growth hormone secretion profiles (Palonge *et al* 1991). This observation was reflected in the studies carried out using a strain of mouse deficient in growth hormone, the little mouse. The difference in levels of microsomal GST expression between the sexes was reversed in the lit/lit strain as compared with the homozygous control. However the precise mechanism of growth hormone action could not be determined from these results.

Future studies would involve the removal of growth hormone by treatment of neonatal animals with monosodium glutamate.Then growth hormone can be artificially administered in order to investigate the processes mediating this effect. Studies are underway at present to analysing the 5'non-coding region of P450 genes regulated by growth hormone, in order to identify the elements responsive to this regulatory control (Mode *et al* 1992). Such studies should be applied to the mouse microsomal GST.

Although the microsomal GST is characterised by its lack of induction of protein expression by xenobiotics, the protein shares a similar pattern of tissue distribution as other drug metabolising enzymes such as P450s and GSTs. The majority of the protein is located in the

liver and the highest expression in an extrahepatic tissue was observed in the lung. The relatively high expression in the lung maybe significant if the role of the enzyme is to protect against oxidative stress.

The use of recombinant expression systems to study the function of the rat microsomal glutathione S-transferase.

The xenobiotic metabolising capacity of the microsomal GST was examined using a heterologous expression system as a model. The merits of such systems have been discussed in length, however there are certain advantages that are directly applicable to the microsomal GST. Expression of the protein in an *in vivo* system would allow the enzyme to be in its natural lipid environment and its putative protective role towards the membrane to be examined. In addition, glutathione conjugates are not necessarily the final product of GST mediated metabolism and it may be the latter metabolites which are responsible for the phenotypic effect of the parent compound. In particular reference to the microsomal GST, it appears that the metabolites produced by a number of different enzyme systems are capable of activating the GST and/or are potential substrates themselves.

S.cerevisiae proved to be the most successful recombinant expression system and was used to study four putative substrates for the microsomal GST. Hexachloro-1,3-butadiene is a particularly well characterised substrate for the microsomal GST and glutathione conjugation leads to the production of toxic thiols (Wolf *et al* 1984; Wallin *et al* 1988; Wolf and Oesch 1989). The expression of the microsomal GST in *S.cerevisiae* under the control of the PGK promoter resulted in an increase in sensitivity to HCBD, which indicated the enzyme was functional *in vivo*. *In vitro* measurements made with the GST model substrate CDNB and using yeast membrane preparations from the yeast expressing the recombinant protein, were lower than expected when considering the level of protein expression. No definite explanation can be put forward for this anomaly, suffice to say that either the protein was inactivated on isolation of the membranes or was partially inactive *in vivo*. However, both *in vivo* and *in vitro* studies demonstrate

conclusively that some of the microsomal GST expressed in the yeast was functional.

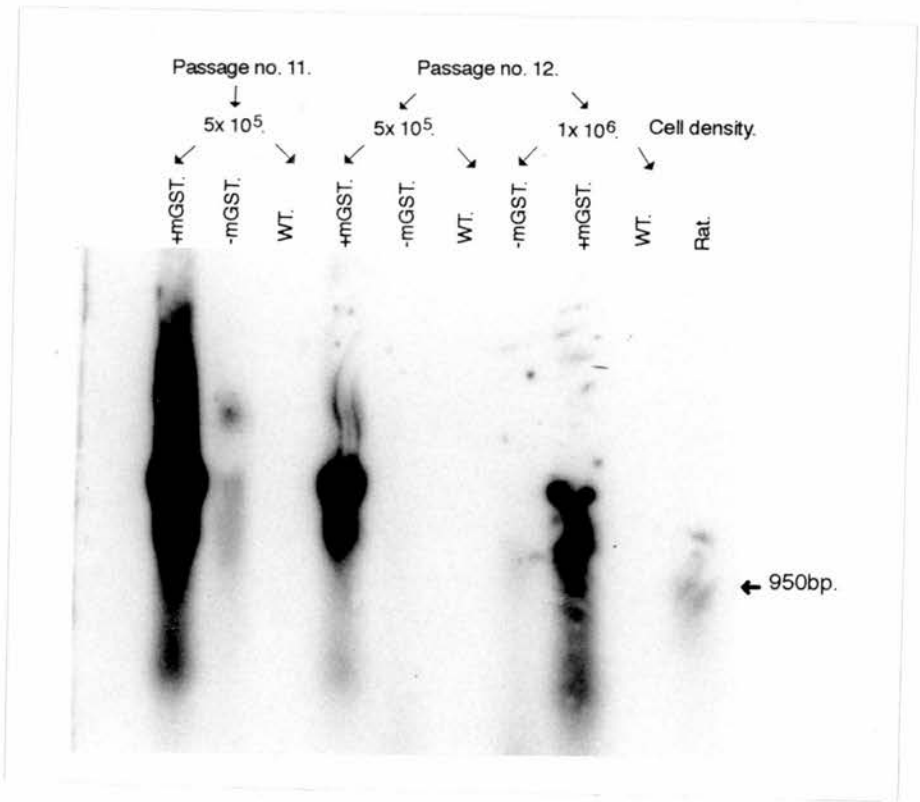
Although 1,2-dibromoethane is not a substrate for the microsomal GST *in vitro* (Carmik *et al* 1991), yeast expressing the recombinant protein were sensitised towards this compound. Previous work suggests that the closely related compound 1,2-dichloroethane is metabolised by the microsomal-located P450 enzymes and it is the resulting metabolite, 1-chloroso-2-chloroethane, which is the substrate for the microsomal GST (Guengerich *et al* 1980). This product of microsomal metabolism is the episulphonium ion, identical to the species produced by the conjugation of the parent compound with glutathione and thought to be responsible for the binding to DNA (van Bladeren *et al* 1980). The experiments with DCE were carried out *in vitro* using preparations of mammalian subcellular fractions and there is no direct evidence to suggest that the same metabolic pathways would occur in yeast. However the recombinant expression system offers the means to express both P450s and the microsomal GST in the same cell and thereby assess the the contribution and interaction of these two enzyme systems in the metabolism of DBE.

Adriamycin was chosen as an agent capable of generating oxidative stress with in the cell. Recently the physiological changes and species generated by this form of toxic insult have been shown to activate the microsomal GST (Wies *et al* 1992). In addition the peroxidase activity of the microsomal GST has suggested a protective role for this enzyme against oxidative stress (Reedy *et al* 1980). However expression of the microsomal GST did not afford protection against adriamycin toxicity. It can of course be concluded that the microsomal GST is in fact not involved in such a role, but oxidative stress is not the only adriamycin-mediated toxicity. DNA intercalation, topoisomerase II inhibition and membrane damage are all manifestations of exposure of cells to adriamycin (Nudd and Wilkie 1983). Further studies could be carried out to investigate the exact nature of toxic insult occurring in yeast and the measurement of such parameters as thiol status and protein thiol loss would be of value. In addition, other compounds capable of generating oxidative stress, such

as menadione and organic peroxides (t-butyl hydroperoxide and cumene hydroperoxide) would help establish definitely whether the microsomal GST can contribute to the protection of the cell against such insult. The focus of much of the studies on this enzyme has been the thiol modification of the protein, and as a result the purpose of the increase in enzyme activity is a major question in understanding the function of the microsomal GST.

The transfection of the cytosolic GST isoenzymes genes into different recombinant expression systems demonstrated that; the ability of these proteins to protect against chemical insult, was to some degree system specific (Table 4.1). The rat microsomal GST was successfully expressed in COS7 cells, under the SV40 promoter, using the pSVL vector (Pharmacia). The Northern blot analysis of COS7 cells transfected with pSVL containing the cDNA in the correct and reverse orientation, pSVL/+mGST and pSVL/-mGST respectively, is shown in Figure 7.1. The quantity of microsomal GST transcript produced under the control of the SV40 promoter was several fold higher than the level observed in rat liver RNA. However the Western blot analysis of whole cell samples of transformed COS7 cells show that the level of microsomal GST protein expression was very low in comparison to the native expression in rat liver (Figure 7.2). The effect of passage number and the cell density at which transfection was performed, were both examined and shown to only marginally influence the level of expression. Although no endogenous microsomal GST was detectable (by western blot analysis) in the COS7 cells there was a high level of cytosolic GST expression, and as a result it was not possible to detect the CDNB activity attributable to the recombinant microsomal GST. However, the expression of the microsomal GST in a mammalian system would offer an alternative model to the yeast for assessing this enzymes role in drug metabolism. The expression of the microsomal GST in yeast under the PGK promoter was maximal at the same point in the growth phase as endogenous resistance to oxidative insult was highest (Jamieson 1992; Collinson and Dawes 1992). As a result this made it hard to asses the role of the expressed protein in protection

Figure 7.1. Northern blot analysis of the rat microsomal glutathione S-transferase in COS7 cells under the control of the SV40 promoter.



10μg of total RNA loaded.

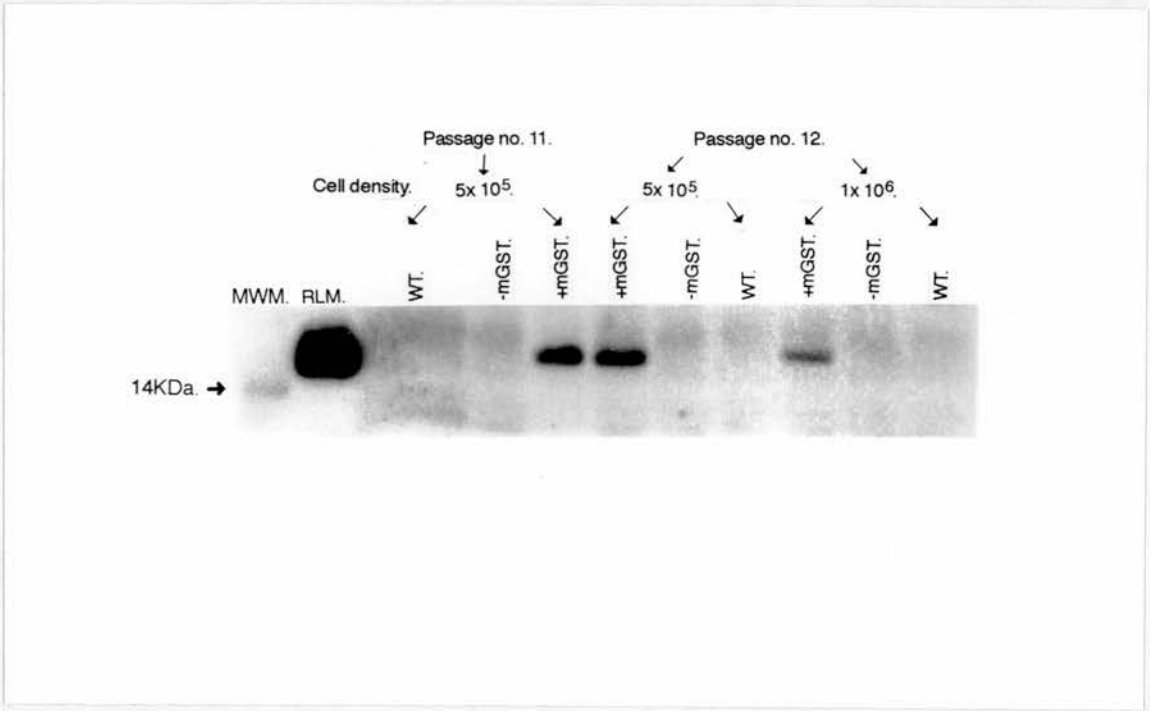
+mGST: rat microsomal GST cDNA in the correct orientation in pSVL.

-mGST: rat microsomal GST cDNA in the reverse orientation in pSVL.

WT: mock transfection.

Cell density: refers to the number of cells/ml at the time of transfection.

Figure 7.2. Western blot analysis of the rat microsomal glutathione S-transferase in COS7 cells under the control of the SV40 promoter.



MWM: molecular weight markers.

10μg of rat liver microsomal protein.

100μg of protein loaded from whole cell extracts of COS7 cells

+mGST: rat microsomal GST cDNA in the correct orientation in pSVL.

-mGST: rat microsomal GST cDNA in the reverse orientation in pSVL.

WT: mock transfection.

Cell density: refers to the number of cells/ml at the time of transfection.

against oxidative stress, however the use of an alternative system, such as a mammalian cell culture model, may help address this question. The employment of a cell line other than the COS7 where all forms of GST are absent or very low, would be required. Also the transfection of the COS7 cells with the pSVL derived constructs was transient and the microsomal GST was only detectable for three days. A number of stable expression vectors exist, such as LK444 which employs a β actin promoter and pCMV4 containing the cytomeglo virus promoter. The development of a cell line in which the microsomal GST was stably expressed and contained low levels of endogenous GST isoenzymes, would provide an alternative model to the *S.cerevisiae* system for such studies.

Initial studies characterising the yeast expression system demonstrated that although the level of rat microsomal GST mRNA produced under the control of the PGK promoter was considerably higher than in the rat liver RNA, the level of protein expressed was only 10-50% of the value observed in the native state. These observations suggest that the message was inefficiently translated in the yeast system. Consequently a number of factors known to influence protein translation were investigated. However no significant increase in translation could be achieved by any of the attempts to optimise the already existing system.

The expression of foreign membrane proteins has been shown to be problematic in a number of systems, so attempts were made to express a truncated form of the microsomal GST. Limited cleavage with trypsin at lysine 41 not only removes the membrane spanning domain, but also increases the activity of the enzyme towards CDNB (Morgenstern *et al* 1990). The resulting recombinant protein was expressed at very low levels and investigation of the quantity of transcript showed that this had been drastically reduced. Interestingly, the truncated protein remained associated with the membrane fractions, which was in keeping with the trypsin treated protein in rat liver microsomes. In an attempt to to raise the level of transcript produced and also increase the stability of the recombinant protein, fusions were made with the N-terminal region of yeast ADH. However the experiment

did not significantly increase the levels of protein expression, but the fusion proteins were all found to be associated with the membrane fractions.

The hydrophobicity plot (Hopp and Wood, Figure 4.17) reveals only one portion of the protein to be hydrophilic, from approximately residue 40-70. The rest of the protein can be divided into three hydrophobic domains (Morgenstern *et al* 1985), the first of which (residues 11-35) has been designated the membrane spanning domain. However the amino sequence does not predict this region to be entirely α helical, since it contains consecutive helix breaking residues at positions 16-18 and 20-23 (TSY and TII, respectively). In addition, a charged residue, lysine, is found at position 25. The studies in which this portion of the protein was replaced with a short stretch of amino acids (4-8 residues) from ADH contained a number of charged residues, but the resulting hybrid proteins still remained with the membrane. It is of course possible that the microsomal GST is not an integral membrane protein, but peripherally associated with the membrane. If this in fact the case this might explain the greater accessibility of the -SH group in the protein to thiol modification over other membrane proteins (Haenen *et al* 1988; Wies *et al* 1992). Although no experiments were carried out to characterise the exact nature of the membrane-association such studies have been conducted on rat liver microsomes (Morgenstern *et al* 1980). Washing microsomes in solutions of high ionic strength, of different pH values or containing chaotropic agents removed less than 10% of the microsomal GST activity. Treatment to remove ribosomes from the endoplasmic reticulum also failed to remove the activity. Soluble proteins associated with the membranes can be removed by washing microsomes on a Sepharose 2B column in 0.15M Tris-HCl pH 8.0 and 10% glycerol, however the microsomal GST could not be extracted from membranes by these means.

Attempts to purify the microsomal GST from other recombinant expression systems demonstrated that the protein had a great tendency to adhere to a number of artificial matrices, and this observation probably also reflects the hydrophobic nature of the protein. Further studies are needed to clarify the exact interaction the microsomal GST

with the membrane environment and this may well illuminate some of the unique activation properties of the protein. The fact that the microsomal GST is reported in a number of membranes suggests that the name "microsomal GST" is some what inaccurate, and if it does posses a general membrane protective role than a more appropriate name would be "the membrane-associated GST."

The highly hydrophobic and basic nature of the microsomal GST was probably instrumental in preventing successful expression of the microsomal GST in *E. coli*. Attempts to express both the full length and truncated rat microsomal GST cDNA failed due to the message not been translated or the protein been unstable in the bacterial environment. Redirection of the protein to the periplasmic space or raising the level of tRNA corresponding to the unfavourable codon usage, AGA/AGG, failed to yield any recombinant protein.

However, expression of the microsomal GST was achieved using the powerful T7 promoter, but the protein was in an inactive form trapped in inclusion bodies. The fact that inclusion bodies are resistant to *E.Coli* proteolytic activity suggests that the failure of prior constructs was due to the instability of the protein when translated. Although it was possible to recover the protein from inclusion bodies in a soluble form no enzyme activity could be measured. This could be due to irreparable damage during solubilisation in Gn-HCl or incorrect folding on entering the non-denaturing environment, which was optimised specifically for the microsomal GST.

Fusion of the microsomal GST to the cytosolic GST from *Schistosoma japonicum* not only reduced the overall hydrophobicity of the protein but reduced the basicity (as judged by the calculation of the pI value of the hybrid protein), there by allowing expression of a soluble stable form of the microsomal GST. In addition fusion proteins have been shown to increase the stability of the readily degraded component (Marston 1986). Although the microsomal GST protein could be proteolytically removed from the carrier protein *in vitro,* the two fragments could not be resolved despite markedly different physical properties of the two GST forms.

On going studies were described in which the microsomal GST

was fused to a more appropriate protein, *E.coli* phosphofructokinase. Although the system had not been fully developed the production of large amounts of active recombinant protein had a specific aim. The catalytic mechanism and activation processes is the most fascinating facet of the microsomal GST and such recombinant DNA technology would allow the investigation of these interesting features.

Bibliography.

Abe A. and Sasaki T. (1989) Sulfhydryl groups in glycolipid transfer protein: formation of an intramolecular disulfide bond and oligomers by Cu^{2+}- catalysed oxidation. BBA **985** 38-44.

Aceto A. Caccuri AM. Sacchetta P. Bucciarelli T. Dragani B. Rosato N. Federici G. Di Ilio C. Dissociation and unfolding of Pi-class glutathione transferase. Biochem.J. **285** (1992) 241-245.

Adang APE. Brussee J. Meyer DJ. Coles B. Ketterer B.van der Gen A. Mulder GJ. Substrate specificity of rat liver glutathione S-transferase isoenzymes for a series of glutathione analogues modified at the γ-glutamyl moiety. Biochem.J. **255** (1988). 721-724.

Adang APE. Brussee J. van der Gen A. Mulder GJ. (1990) The glutathione binding site in the glutathione S-transferases: investigation of the cysteinyl, glycyl and γ-glutamyl domains. Biochem.J. **269** 47-54.

Adang APE. Duindam ALG. Brussee J. van der Gen A. Mulder GJ. van der Gen A. (1988) Synthesis and nucleophilic reactivity of a series of glutathione analogues, modified at the γ-glutamyl moiety. Biochem.J. **255** 715-720.

Adang APE. Meyer DJ. Brussee J. van der Gen A. Ketterer B. Mulder GJ.(1989) Interaction of rat glutathione S-transferase 7-7 and 8-8 with γ-glutamyl- or glycyl-modified glutathione analogues. Biochem.J. **264** 759-764.

Ålin P. Danielsson U. and Mannervik B. (1985) 4-Hydroxyalk-2-enals are substrates for glutathione transferase. FEBS **179** 267-270.

Ames BN. (1979) Identifying enviromental chemicals causing mutuations and cancer. Science **204** 587-593.

Ammerer G. (1983) Expression of genes in yeast using the ADC1 promoter. Methods in Enzymol. **101** 193-201.

Anders MW. Lash.L. Dekant W. Elfra AA. Dohn DR. (1988) Biosynthesis and biotransformation of glutathione S-conjugates to toxic metabolites. CRC Crit. Rev.Toxicol. **18** 311-341.

Andersson C. and Morgenstern R. (1990) Chemical modification of the rat liver microsomal glutathione transferase defines residues important for catalytic function. Biochem.J. **272** 479-484.

Andersson C. Mosialou E. Adang AEP. Mulder GJ. van der Gen A. Morgenstern R. (1991) Studies on the activity and activation of rat liver microsomal glutathione S-transferase with a series of glutathione analogues. J.Biol.Chem. **266** 2076-2079.

Aniya Y. (1989) Activation of the liver microsomal glutathione S-transferase activity by heating. J.Pharmacobio-Dyn. 12 235-240.

Anyia Y and Anders MW. (1989b) Activation of rat liver microsomal glutathione S-transferase by reduced oxygen speices. J.Biol.Chem. **264** 1998-2002.

Anyia Y and Anders MW. (1992) Activation of the rat liver microsomal glutathione S-transferase by hydrogen peroxide: role for protein-dimer formation. Archieves Biochem. Biophys. **296** 611-616.

Anyia Y and Anders MW. (1989a) Regulation of the rat liver microsomal glutathione S-transferase by thiol/disulfide exchange. Archives Biochem.Biophys. **270** 330-334.

Anyia Y. and Anders MW. (1993) Oxidative stress-induced activation of microsomal glutathione S-transferase in rat liver. **45** 37-42.

Armstrong RN. Ammon HL Graminski GR. Zang P. Sessay MA. Kubo Y. Dirkert LA. Mechanistic and structural investigations of glutathione S-transferases. In: Glutathione S-transferases and drug resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. 1990. pg 65-74.

Asano K. Sato H. Aono S. Mizutani N. Watanabe K. Kashiwamata S. (1990) Rat brain microsomal glutathione S-transferase: partial purification and some properties. Biomedical Res. **6** 425-432.

Aswathi YC. Bhatnagar A. and Singh SV. (1987) Evidence for the involvement of histidine at the active site of glutathione S-transferase Y from human liver. Biochem.Biophys.Res.Commun. **143** 965-970.

Azari MR. and Wiseman A. (1982) Purification and characterisation of the cytochrome P-448 component of a Benzo(a)pyrene hydroxylase from Saccharomyces Cerevisiae. Anal.Biochem. **122** 129-138.

Babson JR. Abell NS. Reed DJ. (1981) Protective role of the glutathione redox cycle against adriamycin-mediated toxicity in isolated hepatocytes. Biochem.Pharmcol. **30** 2299-2304.

Balbas P. and Bolivar F. Design and construction of expression plasmid vectors in Escherichia coli. In: Methods in Enzymology 185 (1990) Gene expression technology. Article **3**. pg 14-37.

Barnes HJ. Arotto MP. Waterman MR. (1991) Expression and enzymatic activity of recombinant cytochrome P450 17 a hydroxylase in Escherichia Coli . PNAS **88** 5597-5601.

Bartoszek A and Wolf CR. (1992) Enhancement of doxorubicin toxicity following activation by NADPH cytochrome P450 Reductase. Biochem.Pharm. 43 1449-1457.

Bast A and Haenen GRMM. (1988) Interplay between lipoic acid and glutathione in the protection against microsomal lipid peroxidation. BBA **963** 558-561.

Batist G. Tulpule A. Sinha BK. Katki AG. Myers CE and Cowan KH. (1986) Overexpression of a novel anionic glutathione transferase in multidrug resistant human breast cancer cells. J.Biol.Chem. **261** 15544-15549.

Beamer WG and Eicher EM. (1976) Stimulation of growth in the little mouse J.Endocrinol. **71** 37-45.

Beesley KM. Francis MJ. Clarke BE. Beesley JE. Dopping-Hepenstal PJC. Clare JJ. Brown F. and Romanos MA. (1990) Expression in yeast of the amino-terminal peptide fusions to hepititis B core antigen and their immunological properties. Bio/technology **8** 644-649.

Bellomo G. Mirabelli F. Richelmi P. Orrenius S. (1983) Critical role of the sulfhydrl group(s) in the ATP-dependent Ca^{2+} - sequestration by the plasma membrane fraction from rat liver FEBS **163** 136-139.

Belsham GJ. Barker DG. and Smith AE. (1986) Expression of polyoma virus middle-T antigen

in *Saccharomyces cerevisiae*. Eur.J.Biochem. **156** 413-421.

Bengtsson M. Montelius J. Mankowitz L. and Rydstrom J. (1983) Metabolism of polycyclic aromatic hydrocarbons in the rat ovary: comparison with metabolism in adrenal and liver tissue. Biochem.Pharmcol. **32** 129-136.

Bennetzen JL. and Hall BD. (1982) Codon selection in yeast J.Biol.Chem. **257** 3026-3031.

Benson AM. Batzinger RP. Suh-Yun LO. Bueding E. Cha YN. Talalay P. (1978) Elevation of hepatic glutathione S-transferse activity and protection against mutagenic metabolites of benzo(a)pyrene by dietary antioxidants. Cancer Res. **38** 4486-4495.

Benson AM. Cha YN. Bueding E. Heine HS. Talalay P. (1979) Elevation of extrahepatic glutathione S-transferase and epoxide hydrolase activities by 2(3)-tert-butyl-4-hydroxyanisole. Cancer Res. **39** 2971-2977.

Berstien B. Blanks JL. Smrka AV. Higashijima T. Sternsweis PC. Exton JH. Ross EM. (1992) Reconstitution of Agonist-stimulated phosphaidylinositol 4,5-bisphoshate hydrolysis using purified m1 muscarinic receptor, Gq/11 and phospholipase C-b1. J.Biol.Chem **267** 8081-8088.

Bishop JM. (1987) The molecular genetics of cancer. Science **235** 305-309.

Black SM. BeggsJD. Hayes JD. Bartoszek A. Muramatsu M. Sakai M. Wolf CR. (1990) Expression of human glutathione S-transferases in *Saccharomyces cerevisiae* confers resistance to adriamycin and chlorambucil. Biochem.J. **268** 309-315.

Black SM. Ph.D. Thesis. University of Edinburgh. (1989).

Bligh HFJ. Wolf CR. Smith G. Beggs JD. (1992) Production of cytochrome P450 Reductase yeast-rat hybrid proteins in *Saccharomyces cerevisiae*. Gene **11 0** 33-39.

Bloch CA and Ausubel FM. (1986) Paraquat-mediated selection for mutations in the manganese-superoxide dismutase gene sod A J.Bacteriol. **168** 795-798.

Blow DM. (1971) In: Enzymes 3rd Edition. pg 185-212.

Board PG. and Mannervik B. (1991) The contribution of the C-terminal sequence to the catalytic activity of GST2, a human alpha-class glutathione transferase. Biochem.J. **275** 171-174.

Boost KL. and Black JE. (1986) Molecular characterisation of a corticotropin (ACTH) receptor. Cell Mol. Endocrinol. **44** 1-9.

Booth J. Boyland E. and Sims P. (1961) An enzyme from rat liver catalysing conjugations with glutathione. Biochem J. **79** 516-524.

Botti B. Molsen MT. Trieff NM. Reynolds ES. (1982) Transient decrease of liver cytosolic glutathione S-transferases in rats given 1,2-Dibromoethane. Chem.-Biol. Interactions **4 2** 259-270.

Boyer TD. Vessey DA. Kempner E. (1986) Radiation inactivation of the microsomal glutathione S-transferase. J.Biol.Chem. **261** 16963-16968.

Boyer TD. Zakim D. Vessey DA. (1982) Studies of the inhibitors of microsomal glutathione S-transferase. Biochem.J. **207** 57-64.

Bradford MM. (1976) A rapid sensitive method for the quantitation of microgram quantities of utilizing the principle of protein-dte binding. Anal.Biochem. **72** 248-254.

Brinboim HC and Doly JA. (1979) Rapid extraction procedure for screening recombinant plasmid DNA. NAR **7** 1513-1523.

Brinkman U. Mattes RE. Buckel P. (1989) High-level expression of recombinant genes in Escherichia coli is dependent on the availability of the dna Y gene product. Gene **85** 109-114.

Brown AJP. (1989) Messenger RNA stability in yeast Yeast **5** 239-257.

Buchner J. and Rudolph R. Routes to active proteins from transformed microorganisms. (1991) Current Opinion in Biotechnology **2** 532-538.

Burk RF. (1983) Glutathione-dependent protection by a rat liver microsomal protein against lipid peroxidation. BBA **757** 21-28.

Butler JS. Sadhale PP. Platt T. (1990) RNA processing in vitro produces a variety of *Saccharomyces cerevisiae.* mRNA Mol.Cell.Biol. **6** 2599-2605.

C.Cullin and Pompon. (1988) Synthesis of functional mouse cytochrome P-450 P1 and chemeric P-450 P3-1 in the yeast *Saccharomyces cerevisiae.* Gene **65** 203-217.

Calabresi P. and Parks RE. (1980) Antiproliferative agents and drugs used for immunosuppression, In: The parmacological basis of therapeutics (Eds Gilman AG. Goodman LS. and Gilman A.) MacMillan, New York. pg 1256-1313.

Casalone E. Di Ilio C. Federici G. Polsinelli M. (1988) Glutathione and glutathione metabolising enzymes in yeast. Antonie van Leeuwenhoek **54** 367-375.

Charlton HM. (1984) Mouse mutants as models in endocrine research. Quarterly J.Exp.Physiol. **6 9** 655-676.

Chen Q. Jones TW. Brown PC. Stevens JL. (1987) Antioxidants prevent cysteine conjugate toxicity. Toxicologist **10** 118.

Cheng TC. Beamer WG. Phillips JA. Bartke A. Mallonee RL. and Dowling C. (1983) Etiology of growth hormone deficiency in little Ames and Snell dwarf mice. Endocrinology **11 3** 1669-1678.

Cheng Y-SE. Kowth DY. Kowth TJ. Soltvedt BC. and Zisper D. (1981) Stabilization of a degradable protein by its over expression in *Escherichia coli.* Gene **14** 121-130.

Clapper ML. and Tew KD. (1989) Identificationof a glutathione S-transferase associated with mocrosomea of tumor cells resistant to nitrogen mustards. Biochem.Pharmacol. **38** 1915-1921.

Clapper ML. Buller AL. Smith TM. and Tew KD. (1987) In: Glutathione S-transferases and carcinogenesis (Eds Mantle TJ. Pickett CB. and Hayes JD.) Taylor and Francis, London.

Clark BJ. and Waterman MR. (1991) Heterologous expression of mammalian cytochrome P450 in COS cells. Methods in Enzymology **206** Article 10 pg 100-108.

Clark RG. and Robinson ICAF. (1985) Effects of a fragment of human growth hormone-releasing factor in normal and little mice. J.Endocrinol. **106** 385-392.

Cmarik JL. Inskeep PB. Meredith MJ. Meyer DJ. Ketterer B. Guengerich FP (1991). Selectivity of rat and human glutathione S-transferases in the activation of ethylene dibromide by glutathione conjugation and DNA binding and induction of unscheduled DNA synthesis in human hepatocytes. Cancer Res. **50** 2747-2752.

Collinson LP. and Dawes IW. (1992) Inducibility of the response of yeast cells to peroxide stress. J.Gen.Microbiol. **138** 329-335.

Cotagreave IA. Weis M. Berggren M. Sandy MS. Modleus PW. (1988) Determination of the intracellular protein thiol distribution using monobromobimane derivatisation of intact cells and isolated subcellular fractions. J.Biochem.Biophys.Methods **16** 247-254.

Cousens LS. Shuster JR. Gallegos C. Ku L. Stempien MM. Urdea MS. Sanchez-Pescador R. Taylor A. and Tekamp-Olson P. (1987) High-level expression of proinsulin in Saccharomyces cerevisiae. Gene **61** 265-275.

Cox RA. (1968) The use of guanidinium chloride in isolation of nucleic acids. Methods in Enzymol. **12** 120-129.

Currnette JT. and Babior BM. (1987) In: Advances in human genetics (Eds Harris H and Hirschom K.) Plenum Press, New York pg.16.

D'Ercole AJ. Stiles AD. and Underwood LE. (1984) Tissue concentrations and somatomedin C; Further evidence for multiple sites of synthesis and pancrine or autocrine mechanisms of action. PNAS **81** 935-939.

Dahl J. (1983) Molecular cloning of the gene for phosphofructokinase-2 of Escherichia coli and the nature of a mutation, pfk B1 causing a high level of the enzyme. J.Mol.Biol. **168** 285-305.

Dahlin DC. Miwa GT. Lu AYH. Nelson SD. (1984) N-acetyl-p-benzoquinone imine: a cytochrome P450-mediated oxidation product of acetaminophen. PNAS **81** 1327-1331.

Danielsson UH. and Mannervik B. (1988) Glutathione transferase-structure and catalytic activity. CRC. Crit.Rev.Biochem. **23** 283-337.

Davies KJA (1987) Protein damage and degradation by oxygen radicals: general aspects. J.Biol.Chem **262** 9895-9901.

De Boer HA.Comstock LJ. Vasser M. (1983) The *tac* promoter: a functional hybrid derived from the trp and lac promoters. PNAS. **80** 21-25.

de Montellano PRO. (ed) (1986) Cytochrome P450 structure mechanism and Biochemistry. Plenum Press, London, New York.

Dean RT. (1987) A mechanism for accelerated degradation of intracellular proteins after limited damage by free radicals. FEBS **220** 278-282.

DeJong JL. Mohandas T. and Tu CD. (1990) The gene for the microsomal glutathione S-transferase is on human chromosome 12. Genomics **6** 379-382.

DeJong JL. Morgenstern R. Jornvall H DePierre JW. Tu CD. (1988) Gene expression of rat and human microsomal glutathione S-transferases. J.Biol.Chem. **263** 8430-8436.

Denison MS. Fisher JM. and Whitlock JP. Jr. (1988) The DNA recognition site for the Dioxin-Ah receptor complex. J.Biol.Chem. **263** 17221-17224.

Deshaies RJ. Sanders SL. Feldheim DA. Schekman R. (1991) Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. Nature **349** 806-808.

Dierickx PJ. (1986) Partial purification of the microsomal glutathione S-transferase from rat liver by affinity chromatography. IRCS Med.Sci. **14** 1007-1008.

Ding GJ-F. Lu AHY. and Pickett CB. (1985) Rat liver glutathione S-transferase: nucleotide sequence analysis of a Yb1 cDNA clone and prediction of the complete amino acid sequence of the Yb1 subunit. J.Biol.Chem. **260** 13268-13271.

Dixon KH. Cowell IG. Xia SE. Pemble S. Ketterer B. Taylor JB. (1989) Control of expression of the glutathione S-transferase π gene differs from its rat orthologue. Biochem.Biophys.Res.Comm. **163** 815-822.

Dohn DR. Quebbemann AJ. Borch RF. and Anders MW. (1985) Enzymatic reaction of chlorotrifluroethane with glutahione 19 F NMR evidence for sterochemical control of the reaction. Biochemistry **24** 5137-5143.

Dolan C. Ph.D. Thesis. University of Edinburgh. (1990).

Duan YJ. Komura S. Fisere-Szafarz B. Yagi K. (1988) Purification and characterisation of a novel monomeric glutathione peroxidase from rat liver. J.Biol. Chem. **263** 19003-19008.

Dulik DM. Colvin OM. Fenselau C. (1990) Characterisation of glutathione conjugates of chlorambucil by fast atom bombardment and thermospray liquid chromatography/mass spectrometry. Biomed.Enviro.Mass Spec. **19** 248-252.

Dybig E. Nelson SD. Mitchell JR. Sasame HA. and Gilette JR. (1976) Mol.Pharm **12** 911-920.

Elfra AA. and Anders MW. (1984) Renal processing of glutathione glutathione conjugates. Role in nephrotoxicity. Biochem. Pharmacol. **33** 3729-3734.

Elow I. Modeleus P. Orrenius S. (1984) Oxidation of glutathione during hydrogen peroxide metabolism. A study using rat hepatocytes and the glutathione reductase inhibitor 1,3-bis(chloroethyl)-1-nitosourea. Eur J.Biochem. **138** 459-463.

Elskens M. Penninckx M. Vandeloise R. Vander Donckt E. (1988) Use of a simplex technique and contour diagrams for the determination of the rate reaction constants between glutathione and Thriam in the prescence of NADPH. Int.J.Chem.Kinetics **20** 837-848.

Elskens MT. Jaspers CJ. Penninckx MJ. (1991) Glutathione as an endogenous sulphur source in the yeast Saccharomyces cerevisiae. J.Gen.Microbiol. **137** 637-644.

Estabrook RW. Mason JI. Simpson ER. Peterson JA. Waterman MR. (1991) The heterologous expression of the cytochrome P450: a new approach for the study of enzyme activities and regulation. Adv.Enz.Reg. **31** 365-383.

Fairchild CR. Moscow JA. O'Brien E. Cowan KH. (1990) Multidrug resistance in cells transfected with human genes encoding a variant P-Glycoprotein and glutathione S-transferase p. Mol.Pharmacol. **37** 801-809.

Farriss MW. Pascoe GA. and Reed DJ. (1985) Vitamin E reversal of the effect of extracellular calcium on chemically induced toxicity in hepatocytes. Science **227** 751-754.

Fresht A. Enzyme structure and Mechanism. Second edition. W.H Freeman and company, New York.(1985).

Friedberg T. Bentley P. Stasiecki P. Glatt HR. Raphael D. Oesch F. (1979) The identification, solubilisation and characterisation of microsome-associated glutathione S-transferases. J.Biol.Chem. **254** 12028-12033.

Galiazzo F. Schiesser A. Rotilio G. (1987) Glutathione peroxidase in yeast. Presence of the enzyme and induction by oxidative conditions. Biochem.Biophys.Res.Comm. **147** 1200-1205.

Galiazzo F. Schiesser A. Rotilio G. (1988) Oxygen-independent induction of enzyme activities related to oxygen metabolism in yeast by copper. BBA **965** 46-51.

Garcia GM. Mar PK. Mullin DA. Walker JR. Prather NE. (1986) The *E.Coli* dna Y gene encodes an arginine transfer RNA. Cell **45** 453-459.

Gatenby AG. Viitanen PV. Lorimer GH. (1990) Chaperonin assisted polypeptide folding and assembly: implications for the production of functional proteins in bacteria. Trends in Biotech. **8** 354-358.

Gharyeb J. Kimura H. Takara M. Hsiung H. Masui Y. (1984) Inouye M. Secretion cloning vectors in *Escherichia Coli* . EMBO.J. **3** 2437-2442.

Giachelli CM and Omieciniski CJ. (1987) Developmental regulation of cytochrome P450 genes in rat. Mol.Pharm. **31** 477-484.

Gibson DD. Hornbrook KR. McCay PB. (1980) Glutathione dependent inhibition of lipid peroxidation by a soluble, heat-labile factor in animal tissues. BBA **620** 572-582.

Gietz BD. and Sugino A. (1988) New yeast *Eschericia.Coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74** 527-534.

Goeddel DV. (1990) Systems for heterologous gene expression. Methods in Enzymology **1 8 5** Gene expression technology. Article 1 pg3-10.

Goff SA. and Goodberg AL. (1985) Production of abnormal proteins in *E. coli* stimulates the transcription of lon and other heat shock genes. Cell **41** 587-595.

Gold L. (1990) Expression of Heterologous proteins in *Escherichia Coli.* Methods in Enzymology **185** Gene expression technology. Article 2 pg 11-13.

Gold L. Pribnow D. Schneider T. Shinedling S. Singer BS. Stormo G. (1981) Translation intiation in prokaryotes. Ann.Rev.Microbiol. **35** 365-403.

Gonzalez FJ. Matsunaga T. Nagata K. Meyer UA. Nebert DW. Pastewka J. Kozak CA. Gillette J. Gelboin HV. and Hardwick JP. (1987) Debrisoquine 4-hydroxlase: characterisation of a new P450 gene subfamily, regulation, chromosome mapping and molecular analysis of the DNA rat

polymorphism. DNA **6** 149-161.

Goodman J. and Hochstein P. (1977) Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. Biochem.Biophys.Res.Comm. **7 7** 797-802.

Graminski GF. Kubo Y. Armstrong RN. (1989a) Spectroscopic and kinetic evidence for the thiolate anion of glutathione at the active site of glutathione S-transferase. Biochemistry **28** 3562-3568.

Graminski GF. Zhang P. Mactarr AS.(1989b) Ammon HL. Armstrong RN. Formation of the 1-(S-glutathionyl)-2,4,6-trinitrocyclohexamidienate anion at the active site of glutathione S-transferase: evidence for enzymatic stabilisation of s-complex intermediates in nucleophillic aromatic substistution reactions. Biochemistry **28** 6252-6258.

Grodberg J. and Dunn JJ. (1988) omp T encodes the *Escherichia coli* outer membrane proteases that cleaves T7 RNA polymerase during purification. J.Bacteriol. **170** 1245-1253.

Grunstein M. and Hogness DS. (1975) Colony Hybridisation: a method for the isolation of cloned cDNAs that contain a specific gene. PNAS **72** 3961-3965.

Guarente L. Yocum RR. Gifford P. (1982) A Gal10-CYC1 hybrid yeast promoter identifies the GAL 4 regulatory region as an upstream site. PNAS. **79** 7410-7414.

Guengerich FP. Brian WR. Sari MA. Ross JT. (1991) Expression of mammalian cytochrome P450 enzymes using yeast-based vectors. Methods in enzymology **2 0 6** Article 14 pg 130-145.

Guengerich FP. Crawford WM. Domoradzki JY. Macdonald TL. Watanabe PG. (1980) In vitro activation of 1,2-dichlooethane by microsomal and cytosolic enzymes. Toxicol.Appl.Pharm. **55** 303-317.

Guenthner TM. Nebert DW. and Menard RH. (1979) Microsomal aryl hydrocarbon hydrolase in rat adrenal: regulation by ACTH but not by polycyclic hydrocarbons. Mol.Pharm. **15** 719-728.

Habig WH. Pabst MJ. Jakoby WB. (1974) Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J.Biol.Chem. **249** 7130-7139.

Haenen. G.R.M.M. Tai Tin Tosi J.N.L. Vermeulen N.P.E. Timmerman.H. Bast. A. (1987) 4-Hydroxy-2,3-trans-nonenal stimulates microsomal lipid peroxidation by reducing the glutathione dependent protection. Archieves Biochem.Biophys. **259** 449-456.

Haenen. G.R.M.M. Vermeulen N.P.E. Tai Tin Tosi J.N.L. Regeti HMN. Timmerman.H. Bast. A. (1988) Activation of the microsomal glutathione S-transferase and reduction of the glutathione-dependent protection against lipid peroxidation by acrolein. Biochem.Pharmacol. **3 7** 1933-1938.

Haenen. GRMM. JansenFP. Vermeulen NPE. Bast A. (1991) Activation of the microsomal glutathione S-transferase by metabolites of α-methyldopa. Archieves of Biochem.Biophys. **2 8 7** 48-52.

Hales BF. and Neims AH. (1976) A sex difference in hepatic GST B and the effect of hypophysectomy. Biochem.J. **160** 223-229.

Hallwell RA. Mills R. Tekamp-Olson P. Blacher R. Rosenberg S. Otting F. Masiarz FR. and Scandella CJ. (1987) Amino terminal acetylation of authentic human Cu, Zn superoxide

dismutase produced in yeast. Biotechnology **5** 363-366.

Hammilton R. Wananabe CK. and de Boer HA. (1987) Compilation and comparison of the sequence context around the AUG start codons in Saccharomyces cerevisiae mRNAs. NAR **1 5** 3581-3593.

Hargus SJ. Fitzsimmons ME. Anyia Y. Anders MW. (1991) Sterochemistry of the microsomal glutathione S-transferase catalysed addition of glutathione chlorotrifluoroethane. Biochemistry **30** 717-721.

Harris CM. and Stone WL. (1988) The effects of in vitro lipid peroxidation on the activity of rat liver microsomal glutathione S-transferase from rats supplemented or deficient in antioxidants. Life Sci. **42** 415-420.

Hatayama I. Satoh K. Sato K. (1986) Developemental and hormonal regulation of the major form of hepatic glutathione S-transferase in male mice. Biochem.Biophys.Res.Comm. **140** 581-588.

Hayes JD and Mantle TJ. (1986) The use of immunoblot techniques to descriminate between the glutathione S-transferses Yf, Yk, Yn/Yb and Yc subunits and to study their distribution in extrahepatic tissue. Bichem J. **233** 779-788.

Hayes JD and Wolf CR. (1990) Molecular mechanisms of drug resistance. Biochem.J. **272** 281-295.

Hayes JD. and Wolf CR. Chapter 10: Role of glutathione S-transferases in drug resistance. In: Glutathione conjugation mechanisms and biological significance. (Eds Sies H. and Ketterer B.) Academic press Ltd, New York (1988). pg 315-355.

Hayes PC. Harrison DJ. Bouchier IAD. MeLellan LI. Hayes JD. (1989) Cytosolic and microsomal glutathione transferase isoenzymes in normal human liver and intestinal epithelium. Gut **30** 854-859.

Heidecker G. and Messing J. (1986) Ann.Rev.Plant Physiol. 37 439-466.

Hellinga HW. and Evans PR. (1985) Nucleotide sequence and high level expression of the major *Escherichia Coli* phosphofructokinase. Eur.J.Biochem.**149** 363-373.

Hemminki K and Kallama S. (1986) Reactions of nitrogen mustards with DNA. In: carcinogenicity of alkylating cytostatic drugs (Eds Schmahl D. and Kaldor JM.) IARC Scientific Publications **78** 55-70.

Herminio R. Reisz-Porszasz S. and Hankinson O. (1992) Identification of the Ah receptor nuclear translocator protein (Arnt) as acomponent of the DNA binding form of the Ah receptor. Science **256** 1193-1195.

Herrick D. Parker R. Jacobson A. (1990) Idetification and comparison of stable and unstable mRNAs in Saccharomyces cerevisiae. Mol.Cell Biol. **10** 2269-2284.

Hill KE and Burk RF. (1984) Influence of vitamin E and selinium on glutathione-dependant protection against microsomal lipid peroxidation. Biochem.Pharmacol. **33** 1065-1068.

Hirono I. (1960) Non-protein sulphyhryl group in the original strain and sub-line of the ascites tumour resistant to alkylating reagents. Nature **186** 1059-1060.

Hoekema A. Kastelein RA. Vasser M. DeBoer HA. (1987) Codon replacement in the PGK 1 gene in *Saccharomyces cerevisiae* : experimental approach to study the role of biased codon usage in gene expression. Mol.Cell.Biol. **7** 2914-2924.

Hoesch RM. and Boyer TD. (1989) Localisation of a portion of the active site of two rat liver glutathione S-transferases using a photoaffinity label. J.Biol.Chem. **264** 17712-17717.

Homma H. Maruyama H. Niitsu Y. and Listowsky I (1986) A subclass of glutathione S-transferases as intracellular high-capacity and high-affinity steriod-binding proteins. Biochem.J. **235** 763-768.

Hornsby PJ. (1988) The mechanism and action of ACTH in the adrenal cortex. In: Hormones and their action part II (Eds. Cooke BA. King RJB. Van der Molen HJ.) Elsevier Science Publishers BV (Biomedical Division), Amsterdam.

Igarashi T. Satoh T. Iwashita K. Ono S. Ueno K. and Kitagawa H. (1985) Sex- difference in subunit composition of hepatic glutathione S-transferase in rats. J. Biochem. **98** 117-123.

Igarashi T. Satoh T. Ueno K. and Kitagawa H. (1983) Sex-related difference in the hepatic glutathione level and related enzyme activities in rat. J.Biochem. **93** 33-36.

Ikeda T. Alteri M. Chen Y-T. Nakamura N. Tukey RH. Nebert DW. and Negishi M. (1983) Characterisation of P2-450 (20S) mRNA. Association with the P1-P450 genomic gene and differential response to the inducers 3-methylcholanthrene and isosaferole. Eur.J.Biochem **1 3 4** 13-18.

Itoh H. Fukada Y. Morata K. and Kimara A. (1983) A transformation of intact yeast cells treated with alkali cations. J.Bacteriol. **153** 163-168.

Jabbar MA. Sivasubramanian N. and Nayak DP. (1985) Influenza viral (A/WSN/33) hemaglutinin is expressed and glycosylated in *Saccharomyces cerevisiae*. PNAS **82** 2019-2023.

Jaesche H and Mitchell JR. (1989) Mitochondria and xanthine oxidase both generate reactive oxygen species in isolated perfused rat liver after hypoxic injury. Biophys.Res.Commun. **160** 140-147.

Jamieson D. (1992) *Saccharomyces cerevisiae* has a distinct adaptive responses to both hydrogen peroxide and menadione. J.Bacteriol. **174** 6678-6681.

Janowicz ZA. Hening U. and Hollenberg CP. (1982) Synthesis of Escherichia coli outer membrane protein Omp A in yeast. Gene **20** 347-358.

Jaspers C and Penninckx M. (1984) Glutathione metabolism in the yeast Saccharomyces cerevisiae. Evidence that γ-glutamyltranspeptidase is a vacuolar enzyme. Biochemie **66** 71-74.

Jaspers C. Gigot D. and Penninckx M. (1985) Pathways of glutathione degredation in the yeast *Saccharomyces cerevisiae*. Phytochemistry **24** 703-707.

Jornvall H. and Persson B. The thioredoxin/glutaredoxin family compared to those of different glutathione transferases and other proteins. Thioredoxin and glutaredoxin systems: structure and function (Eds Holemgren A.) Raven press, New York. (1986).

Jowett T. Wajidi MFF. Oxtoby E. and Wolf CR. (1991) Mammalian genes expressed in

Drosophila: a transgenic model for the study mechanisms of chemical mutagenesis and metabolism. EMBO. J. **10** 1075-1081.

Kagawa N. and Waterman MR. (1990) cAMP-dependent transcription of the human CYP21B (P450 c21) gene requires a cis-regulatory element distinct from the consensus cAMP-regulatory element. J.Biol.Chem. **265** 11299-11305.

Kalb VF. Loper JC. Dey CR. Woods CW. Sutter TR. (1986) Isolation of a cytochrome P-450 structural gene from *Saccharomyces cerevisiae.* Gene **45** 237-245.

Kapelli O. (1986) Cytochrome P450 in yeasts. Microbiol.Rev. **50** 244-258.

Kelly D. and Parry JM. (1983) Metabolic activation of cytochrome P-450/P-448 in the yeast *Saccharomyces cerevisiae.* Mut.Res. **108** 147-159.

Kelly SL. Kelly DM. King DJ. Wiesman A. (1985) Interaction between yeast cytochrome P450 and chemical carcinogens. Carcinogenesis **6** 1321-1325.

Ketterer B. and Meyer DJ. (1989) Glutathione transferases: a possible role in the detoxification and repair of DNA and lipid hydroperoxides. Mut.Res. **214** 33-40.

Ketterer B. Meyer DJ. Clark AG. Chapter 3.Soluble glutathione transferase isoenzymes. In: Glutathione conjugation mechanisms and biological significance. (Eds Sies H. and Ketterer B.) Academic press Ltd, New York (1988) pg 74-135.

Ketterer B. Meyer DJ. Taylor JB. Pemble S. Coles B. Fraser G. GSTs and protection against oxidative stress. In: Glutathione S-transferases and drug resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. 1990. pg 97-109.

Kimura S. Gonzales FJ. Nedert DW (1984) The murine Ah locus. J.Biol.Chem. **259** 10705-10713.

Kingsman SM. Cousens D. Stanway CA. Chambers A. Wilson M. Kingsman AJ. (1990) High efficiency yeast expression vectors baised on the promoter of the phosphoglycerate kinase gene. Methods in Enzymology **185** Gene expression technology. Article 27 pg 320-329.

Kinskern PJ. Hagopian A. Montgomery DL. Burke P. Dunn NR. Hofman KJ. Miller WJ. and Ellis RW. (1986) Unusually high-level expression of a foriegn gene (hepatitis B virus core antigen) in *Saccharomyces cerevisiae.* Gene **46** 135-141.

Kittomoto K. Yoshizawa K. Ohsumi Y. Anraku Y. (1988) Dynamic aspects of a vacoular and cytosolic amino acid pools of *Saccharomyces cerevisiae.* J.Bacteriol. **170** 2683-2686.

Kohno T. Carmichael DF. Sommer A. Thompson RC. (1990) Refolding of recombinant proteins. Methods in Enzymology **185** Gene expression technology. Article 16 pg 187-189.

Koob M. and Dekant W. (1991) Bioactivation of xenobiotics by the formation of toxic glutathione conjugates. Chem.-Biol.Int. **77** 107-136.

Kosower NS. and Kosower EM. (1978) The glutathione status of cells. Int.Rev.Cytology. **54** 109-160.

Kotewicz ML. D'Alessio JM. Driftmier KM. Blodgett KP. and Gerad GF. (1985) Cloning and overexpression of Moloney murine leukemia virus reverse transcriptase in *Escherichia coli*. Gene **35** 249-258.

Kozak M. (1984) Selection of intiation sites by eukaryotic ribosomes: effect of inserting random AUG triplets upstream from the coding sequence of preproinsulin. NAR **1 2** 3873-3893.

Kramer RA. Zakher J. Kim G. (1988) Role of the glutathione redox cycle in aquired and de novo multidrug resistance. Science **241** 694-696.

Krause J. Hay R. Kowollik C. Brdiczka D. (1986) Cross-linking analysis of the yeast outer mitochondrial membrane. BBA **860** 690-698.

Kumagai H. Tamaki H. Koshino Y. Suzuki H. Tochikura T. (1988) Distribution, formation and stabilization of yeast glutathione S-transferase. Agric.Biol.Chem. **52** 1377-1382.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227** 680-685.

Lamartiniere CA. (1981) The hypothalmic-hypophyseal-gonadal regulation of hepatic glutathione S-transferases in the rat. Biochem. J. **198** 211-217.

Lands WEM. and Pendleton RB. (1988) In: Oxygen radicles in biology and medicine. (Eds Simic MG. Taylor KA. Ward JF. and von Sonntag C.) Plenum Press, New York. pg. 675.

Larsen GL. and Stevens JL. (1985) Microfloral cysteine conjugate b-lyase: an enzyme responsible for the formation of xenobiotic thiols in the gastrointestinal tract. Microecology and Therapy. **15** 261-266.

Larson JR. Coon MJ. and Porter TD. (1991) Alcohol-inducible cytochrome P450IIE lacking the hydrophobic NH2-terminal segment retains catalytic activity and is membrane bound when expressed in *Escherichia coli*.J.Biol.Chem. **266** 7321-7324.

Lash LH. and Anders MW. (1987) Mechanism of action of S-(1,2-dichloro-vinyl)-L-cysteine and S-(1,2-dichloro-vinyl)-L-homocysteine-induced renal mitochondrial toxicity. Mol.Pharm. **32** 549-556.

Lau FT-K. Frescht AR. Hellinga HW. and Evans PR. (1987) Site-directed mutagenesis in the effector site of *Escherichia coli* phosphofructokinase. Biochemistry **26** 4143-4148.

Le Bras G. and Garel J-R. (1982) A proteolyzed derivative of *Escherichia coli* phosphofructokinase is no longer sensitive to allosteric effectors and still shows cooperativity in substrate binding. Biochemistry **21** 6656-6660.

Le Quoc K. Le Quoc D. (1985) Crucial role of sulfhydryl groups in the mitochondrial inner membrane structure. J.Biol.Chem. **260** 7422-7428.

Lehninger AL. Verces A. Bababunmi E. (1978) Regulation of Ca^{2+} from mitochondria by the reduced state of pyridine nucleotides PNAS **75** 1690-1694.

Lewis AD. Hayes JD. and Wolf CR. (1988) Glutathione and glutathione dependant enzymes in adenocarcinoma cell lines derived from a patient before and after the onset of drug resistence: intrinsic differences and cell cycle effects. Carcinogenesis **9** 1283-1287.

Leyland-Jones BR. Townsend AJ. Tu CD. Cowan.KH. Goldsmith ME. (1991) Antineoplastic drug sensitivity of human MCF-7 Breast Cancer cells stabily transfected with a human a class glutathione S-transferase gene. Cancer Res. **51** 587-594.

Li Y. and Liberman MW. (1989) Two genes associated with liver cancer are regulated by different mechanisms in rasT24 transformed liver epithelial cells. Oncogene **4** 795-798.

Li Y. Seyama T. Godwin AK. Winokur TS. Lebovitz RM. and Liberman MW. (1988) MTrasT24, a metallothionein-ras fusion gene, modulates expression in cultured rat liver cells of two genes associated with in vivo liver cancer. PNAS. **85** 344-349.

Lindsay DF. Mullin DA. Walker JR. (1989) Characterisation of the cryptic lamboid prophage DLP12 of *Escherichia coli* and overlap of the DLP12 intergrase gene with the tRNA gene arg U. J.Bacteriol. **171** 6197-6205.

Liochev SI and Fridovich I. (1991) Effects of overproduction of superoxide dismutase on the toxicity of paraquat toward *Escherichia coli*. J.Biol.Chem **266** 8747-8750.

Loison G. Vidal A. Findeli A. Roitsch C. Balloul JM. and Lemoine Y. (1989) High level expression of a protective antigen of schistosomes in *Saccharomyces cerevisiae*. Yeast **5** 497-507.

Looman AC. Bodlaender J. Comstock LJ. Eaton D. Jhurani.P DeBoer HA. van Kippenberg PH. (1987) Influence of the codon following the AUG intiating codon on the expression of a modified lac Z gene in *Escherichia Coli*. EMBO.J. **6** 2489-2492.

Losson R. and Lacoute F. (1979) Interference of mutations with eukaryote messenger RNA stability. PNAS **76** 5134-5137.

Lowry OH. Rosenbrough NJ. Farr AL. and Randall RJ. (1951) Protein measurements with the folin phenol reagent. J.Biol.Chem. **193** 265-275.

Lu AHY and West SB. (1980) Multiplicity of mammalian cytochrome P450. Pharmacol.Rev. **3 1** 277-295.

Lu FT. Fresht AR. Hellinga HW. Evans PR. (1987) Site-directed mutagenesis in the effector site of Escherichia Coli phosphofructokinase. Biochemistry **26** 4143-4148.

Lund J. Ahlgren R. Wu DH. Kagimoto M. Simpson ER and Waterman MR. (1990) Transcriptional regulation of the bovine CYP17 (P450 17α) gene: identification of two cAMP regulatory regions lacking the consensus cAMP-responsive element (CRE). J.Biol.Chem. **2 6 5** 3304-3312.

Lundqvist G. and Morgenstern R. (1992) Mechanism of activation of the rat liver microsomal glutathione transferase by noradrenaline and xanthine oxidase. Biochem.Pharmacol. **43** 1725-1728.

Lundqivist. G and Morgenstern R. (1992) Studies on the activation of the rat liver microsomal glutathione transferase in isolated hepatocytes. Biochem.Pharmacol. **43**. 131-135.

Maniatis T. Fritsch EF. and Sambrook J. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, New York.

Mankowitz L. Castro VM. Mannervik B. Rydstrom J. DePierre JW. (1990) Increase in the amount of glutathione S-transferase 4-4 in the rat adrenal gland after hyphysectomy and down

regulation by subsequent treatment with adrenocorticotrophic hormone. Biochem.J. **265** 147-154.

Mankowitz L. DePierre JW. Mannervik B. Hansson HA. (1991) Immunohistochemical distribution of the isoenzymes of glutathione transferase in adult rat adrenal gland before and after hypophysectomy. Biochem.J. **280** 399-405.

Mankowitz L. Rydstrom J. DePierre JW. (1991) Adrenocorticotropin-dependent regulation of glutathione transferase subunit 4 in cultured rat adrenal cells. Eur.J.Biochem. **200** 89-93.

Mannervik B. (1986) Glutathione and the evolution of enzymes for the detoxification of products of oxygen metabolism. Chemica.Scripta. **26B** 281-284.

Marchand DH. Reed DJ. (1989) Identification of the reactive glutathione conjugate S-(2-chloroethyl)glutathione in the bile of 1-bromo-2-chloroethane-treated rats by high pressure liquid chromatography and precolumn derivatization with o-phthalaldehyde. Chem.Res.Toxicol. **2** 449-454.

Marston FAO. (1986) The purification of eukaryotic polypeptides synthesised in Escherichia coli . Biochem.J. **240** 1-12.

Masui Y. Mizuno T. Inouye M. (1984) Novel high level expression cloning vechiles: 104 amplification of *Escherichia Coli* minor protein. Biotecnology **2** 81-85.

Masukawa T. and Iwata H. (1986) Possible regulation of the microsomal glutathione S-transferase activity in rat liver. Biochem. Pharmacol. **35** 435-438.

McCay PB. Gibson DD. Fong KL. Hornbrook KR. (1976) Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. BBA **431** 459-468.

McLellan LI and Hayes JD. Differential regulation of murine glutathione S-transferases by xenobiotics. In: Glutathione S-transferases and drug resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. 1990. pg 196-210.

McLellan LI and Hayes JD. (1987) Sex constitutive expression of the preneoplastic marker glutathione S-transferase, YfYf, in mouse liver. Biochem.J. **245** 399-406.

Meehan RR. Forrester LM. Stevenson K. Hastie ND. Buchmann A. Kunz HW. and Wolf CR. (1988) Regulation of phenobarbital-inducible cytochrome P450s in rat and mouse liver following dexamethasone treatment and hypophysectomy. Biochem J. **245** 789-797.

Mellor J. Dobson MJ. KIngsman AJ. Kingsman SM. (1987) A transcriptional activator is located in the coding region of the PGK gene. NAR. **15** 6243-6259.

Mellor J. Dobson MJ. Roberts NA. Tuite MF. Emtage JS. White S. Lowe PA. Patel T. Kingsman AJ. Kingsman SM. (1983) Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. Gene **24** 1-14.

Merideth MJ. amd Reed DJ. (1982) Status of mitochondrial glutathione in the isolated hepatocyte. J.Biol.Chem. **257** 3747-3753.

Messenguy F. Colin D. and Ten Have JP. (1980) Regulation and compartmentation of amino acid pools in *Saccharomyces cerevisiae* and affects on metabolic control. Eur.J.Biochem. **108** 439-447.

Meyer DJ. and Ketterer B. (1982) 5α,6α-Epoxy-cholesteran-3β-ol (cholesterol α-oxide): a specific substrate for rat liver glutathione transferase B. FEBS Lett. **150** 499-502.

Meyer DJ. Coles B. Pemble SE. Gilmore KS. Fraser GM. and Ketterer B. (1991) Theta, a new class of glutathione transferase from rat amd man. Biochem.J. **274** 409-414.

Miester A. and Anderson ME. Glutathione. (1982) Annu.Rev.Biochem. **52** 711-760.

Mimnaugh EG. Kenndy KA. Trush MA. Sinha BK. (1985) Adriamycin-enhanced membrane lipid peroxidation in isolated rat nuclei. Cancer Res. **45** 3296-3304.

Mimnaugh EG. Trush MA. Bhatnagar M. Gram TE. (1985) Enhancement of reactive oxygen-dependent mitochondrial membrane lipid peroxidation by the anticancer drug adriamycin. Biochem.Pharmacol. **34** 847-856.

Mirribelli F. Salis A. Vairetti M. Bellomo G. Thor H. Orrenius S. (1989) Cytoskeletal alterations in human platlets exposed to oxidative stress are mediated by oxidative stress are mediated by oxidative and Ca^{2+} mechanisms. Arch.Biochem.Biophys. **270** 478-488.

Mode A. Tollet P. Strom A. Legraverend C. Liddle C. Gustafsson JA. (1992) Growth hormone regulates the expression of P450 in the rat. Advan.Enzyme.Regul. **32** 255-263.

Moffatt BA. and Studier FW. (1987) T7 lysozyme inhibits transcription by T7 RNA polymerase. Cell **49** 221-227.

Molialou E. and Morgenstern R. (1989) Activity of the rat liver microsomal glutahthione S-transferase towards products of lipid peroxidation and studies of the effect of inhibitors on glutathione-dependent protection against lipid peroxidation. Archieves of Biochem. Biophys. **275** 289-294.

Molsialou E and Morgenstern R. (1990) Inhibition studies on rat liver microsomal glutathione S-transferase. Chem.-Biol. Interactions. **74** 275-280.

Moore M. Thor H. Moore G. Nelson S. Modleus P. Orrenius S. (1985) The toxicity of acetaminophen and N-acetyl-p-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca^{2+}. J.Biol.Chem. **260** 13035-13040.

Moore RE. Davies MS. O'Connell KM. Harding EI. Wiegand RC. Tiemeier DC. (1986) Cloning and expression of a cDNA encoding a maize glutathione S-transferase in *E.coli*. NAR **14** 7227-7235.

Morgenstern R. Lundquist Jornvall H DePierre JW. (1989) Activation of the rat liver microsomal glutathione S-transferase by limited proteolysis. Biochem.J. **260** 577-582.

Morgenstern R. and DePierre JW. (1988) Chapter 3. Membrane-bound glutathione transferase. In: Glutathione conjugation mechanisms and biological significance. (Eds Sies H. and Ketterer B.) Academic press Ltd, New York . pg 157-174.

Morgenstern R. and DePierre JW. (1985) Microsomal glutathione transferase. Rev.Biochem.Toxicol. **7** 67-104.

Morgenstern R. and DePierre JW. (1983a) Microsomal glutathione transferase: purification in unactivated form and further characterisation of the activation process, substrate specificity and amino acid composition. Eur.J.Biochem. **134** 591-597.

Morgenstern R. DePierre JW. and Ernster L. (1979) Activation of the microsomal glutathione S-transferase by sulfydryl reagents. Biochem.Biophys. Res. Comm. **87** 657-663.

Morgenstern R. DePierre JW. and Ernster L. (1980) Reversible activation of the rat liver microsomal glutathione S-transferase activity by 5,5'-dithiobis(2-nitrobenzoic acid) and 2,2' dipyridyl disulphide. Acta.Chem.Scand. **B34** 229-230.

Morgenstern R. DePierre JW. Jornvall H. (1985) Mirosomal glutathione transferase: primary structure. J.Biol.Chem **260** 13976-13983.

Morgenstern R. Guthenberg C. DePierre JW. (1982a) Microsomal glutathione transferase: purification, intial characterisation and demonstration that is not identical to the cytosolic glutathione S-transferases A,B and C. Eur.J.Biochem. **128** 243-248.

Morgenstern R. Guthenberg C. Mannervik B. DePierre JW. (1983b) The amount and nature of glutathione transferases in rat liver microsomes determined by immunological methods. FEBS **160** 264-268.

Morgenstern R. Lundqvist G. Andersson G. Balk L. DePierre JW. (1984) The distribution of microsomal glutathione transferase amongst different organelles, different organs and different organisms. Biochem.Pharm. **33** 3609-3614.

Morgenstern R. Lundqvist G. Hancock V. DePeirre JW. (1988) Studies on the activity and activation of the rat liver microsomal glutathione S-transferase, in particular with a substrate analogue series. J.Biol.Chem. **263** 6671-6675.

Morgenstern R. Lundqvist G. Jornvall H. DePierre JW. (1989) Activation of rat liver microsomal glutathione transferase by limited proteolysis. Biochem.J. **260** 577-582.

Morgenstern R. Lundqvist G. Mosialou E. Andersson C. (1990). Membrane-bound glutathione S-transferase: function and properties. In: Glutathione S-transferases and drug resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. pg 57-64.

Morgenstern R. Meijer J. DePierre JW. Ernster L. (1980) Characterisation of the rat liver microsomal glutathione S-transferase activity. Eur.J.Biochem. **104** 167-174.

Morgenstern R. Wallin H. DePierre J.W. (1990) Mechanisms of activation of the microsomal glutathione transferase. In: Glutathione S-transferases and drug resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. pg 57-46.

Morrow CS. Goldsmith ME. Cowan KH. (1990) Regulation of human glutathione S-transferase π gene transcription: influence of 5'-flanking sequences and trans-activating factors which recognise the AP-1-binding site. Gene **88** 215-255.

Moscow JA. Townsend AJ. Cowan. KH. (1989) Elevation of π class glutathione S-transferase activity in human breast cells by transfection of the GST π gene and its effects to sensitivity to toxins. Mol.Pharm **36** 22-28.

Mozhaev MW. Berezin IV. Martinek K. (1988) Structure-stability relationship in prteins: fundemental tasks and strategy for the developement of stabilised enzyme catalsis for biotechnology. CRC Crit. Rev. Biochem. **23** 235-281.

Mulder GJ. Adang AEP. Brussee J. Ketterer B. Meyer D. van der Gen A. (1990) The glutathione binding site of the glutathione S-transferase isoenzymes from the rat: selectivity towards tripeptide analogues of glutathione. In: Glutathione S-transferases and drug

resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. pg 75-84.

Munro S. and Pelham HR A (1987) C-terminal signal prevents secretion of luminal ER proteins. Cell **48** 899-907.

Murty CVR. Rao KVS. Chung KW. and Roy AK. (1987) Independent regulatory influence of growth hormone on the hepatic synthesis of a2u-globulin. Endocrinology **121** 1819-1823.

Nagasaka Y. Fujii S. Kaneko T. (1989) Microsomal glutathione-dependant protection against lipid peroxidation acts through a factor other than glutathione peroxidase and glutathione S-transferase in rat liver. Archeives Biochem.Biophys. **274** 82-86.

Naggli B. Freiderich DH. Wurgler FE. (1986) Endogenous promutagen activation in the yeast *Saccharomyces cerevisiae*: factors influencing Aflatoxin B1 mutagenicity. Mut.Res. **175** 223-229.

Nakagawa K. Saijo N. Shigeki T. Sakai M. Tsunokawa Y. Yokota J. Muramatsu M Sato K. Terada M. Tew KD. (1990) Glutathione S-transferase p as a determinant of drug resistance in transfected cell lines. J.Biol.Chem. **265** 4296-4301.

Nguyen T. and Pickett CB. (1992) Regulation of the rat glutathione S-transferase Ya subunit gene expression: Dna-protein interaction at the antioxidant responsive element. J.Biol.Chem. **267** 13535-13539.

Nicotera P. Hartzell P. Baldi C. Sevensson S. Bellomo G. Orrenius S.(1986) Cystamine induces toxicity in hepatocytes through the elavation of cytosolic Ca^{2+} and the stimulation of a nonlysoaomal proteolytic syatem J.Biol.Chem. **261** 14628-14635.

Nilsson B. and Anderson S. (1991) Proper and improper folding of proteins in the cellular enviroment. Annu.Rev.Microbiol. **45** 607-635.

Nishino H. and Ito A. (1989a) Increase in glutathione disulphide level regulates the activity of the microsomal glutathione S-transferase in rat liver. Biochem.Int. **19** 731-735.

Nishino H. (1989) Effect of Fraction V powder from bovine serum on rat liver microsomal glutathione S-transferase activity. Biochem.International. **18** 1269-1277.

Nudd R. and Wilkie D. (1983) Chemical carcinogens, mitochondria and glutathione. Chemistry in Britain 911-913.

Oesch F. and Wolf CR. (1989) Properties of the microsomal and cytosolic glutathione S-transferases involved in hexachloro-1:3-butadiene conjugation. Biochem.Pharmcol. **38** 353-359.

Okuda A. Imagawa M. Maeda Y. Sakai M. and Muramatsu M. (1989) Structural and functional analysis of an enhancer GPE1 having a phorbol 12-O-tetradecanoate 13-acetate responsive element-like sequence found in the rat. J.Biol.Chem. **264** 16919-16923.

Okuda M. Ikai I. Chance B. and Kumar C. (1991) Oxygen radical production during ischemia-reperfusion in the isolated perfused rat liver as monitored by luminol enhanced chemiluminescence. Biochem.Biophys.Res.Commun. **176** 217-221.

Olafsdotti K. Pascoe GA. Reed DJ. (1988) Mitochondrial glutathione status during Ca^{2+} ionophore-induced injury to isolated hepatocytes. Arch.Biochem.Biophys. **263** 226-235.

Olson RD. Boerth RC. Gerber JG. Nies AS. (1981) Mechanism of adriamycin cardiotoxicity: evidence for oxidative stress. Life Sci. **29** 1393-1401.

Orrenius S. (1985) Biochemical mechanisms of cytotoxicity. TIPS **FEST** supplement S15-S20.

Ozawa N. and Guengerich FP. (1983) Evidence for the formation of an S-[2-(N7-guanyl)ethyl]glutathione adduct in glutathione mediated binding of the carcinogen 1,2-dibromoethane to DNA. PNAS **80** 5266-5270.

Packer L. Maguire JJ. Mehlhorn RJ. Serbinova E. Kagan VE. (1989) Mitochondrial and microsomal membranes have a free radical reductase activity that prevents chromanoxyl radical accumulation. Bichem.Biophys.Res.Comm. **159** 229-235.

Packer L. Maguire JJ. Melhorn RJ. Serbinova E. Kagan VE. (1989) Mitochondrial and microsomal membranes have a free radical reductase activity that prevents chromanoxyl radical formation. Biochem.Biophys.Res.Comm. **159** 229-235.

Pampori NA. Agarawal AK. Waxman DJ. Shapiro BH. (1991) Differential effects of neonatally administered glutamate on the ultradian pattern of circulating growth hormone regulating expression of sex-dependent forms of cytochrome P450. Biochem.Pharmacol. **41** 1299-1309.

Park E-M and Thomas JA. (1988) S-thiolation of creatine kinase and glycogen phosphorylase b intiated by partially reduced oxygen species. BBA **964** 151-160.

Parlong FP. Miell JP. Corder R. Gaillard RC. (1991) Dexamethasone treatment in man induces changes in 24-hour growth hormone (GH) secretion profile without altering total GH relase. J.Clin.Endo.Metab. **73** 1191-1196.

Pascoe GA. and Reed DJ. (1987) Vitamin E protection against chemical-induced cell injury. Archives Biochem.Biophys. **256** 159-166.

Pelham HRB. (1990) The retention signal for soluble proteins of the endoplasmic reticulum. TIBS 15 483-486.

Penninckx M. Jaspers C. and Legrain M. (1983) The glutahione-dependent glyoxylase pathway in *Saccharomyces cerevisiae*. J.Biol.Chem. **258** 6030-6036.

Pesly F. and Lacoute F. (1984) Effect of ochre nonsense mutations on yeast URA 1 mRNA stability. Current Genetics **8** 277-282.

Peterson GB. Stockwell PA. Hill DF. (1988) Messenger RNA recognition in Escherichia Coli : a possible second site of interaction with 16S ribosomal RNA. EMBO.J. **7** 3957-3962.

Pharmacia. Afinity chromatography:- principles and methods. (1983).

Piccolomini R. Di Ilio C. Aceto A. Allocanti N. Faraone A. Cellini L. Ravagnan Federici G. (1989) Glutathione S-transferase in bacteria: subunit composition and antigenic characterization. J.Gen.Microbiol. **135** 3119-3125.

Pickett CB. and Lu AYH. (1989) Glutathione S-transferases: gene structure, regulation and biological function. Annu.Rev.Biochem. **64** 743-764.

Porter TD. and Larson JR. (1991) Expression of mammalian cytochrome P450 in Escherichia

coli. Methods in Enzymology **206** Article 11 pg 108-116.

Power C. Sinha S. Webber C. Manson MM. and Neal GE. (1987) Transformation related expression of glutathione S-transferase P in rat liver cells. Carcinogenesis **8** 797-802.

Principto GB. Daneilsson UH. Mannervik M. (1988) Relaxed thiol sustrate specificity of glutathione transferases effected by a non-substrate glutathione derivative. FEBS **231** 155-158.

Puchalski RB. and Fahl WE. (1990) Expression of recombinant glutathione S-transferase π, Ya or Yb1 confers resistance to alkylating agents. PNAS. **87** 2443-2447.

Puri NK. Crivelli E. Cardamone M. Fiddes R. Bertolini J. Ninham B. Brandon MR. (1992) Solubilization of growth hormone and other recombinant proteins from *Escherichia Coli* inclusion bodies by using a cationic surfacant. Biochem.J. **285** 871-879.

Purvis IJ. Bettany AJE. Loughlin L. and Brown AJP. (1987) Translation and stablility of an *Escherichia coli* β-galactosidase mRNA expressed under the control of pyruvte kinase sequences in *Saccharomyces cerevisiae*. NAR **15** 7963-7974.

Pyerin W. Taniguchi H. Horn F. Oesch F. Amelizad Z. Friedberg T. Wolf CR. Isoenzyme specific phospkorylation of cytochrome P-450 and other drug metabolising enzymes. (1987) Biochem.Biophys.Res.Comm. **142** 885-889.

Reed DJ. Brodie AE. Meredith MJ. (1983) Cellular heterogenity in the status and functions of cysteine and glutathione. In: functions of glutathione: biochemical, physiological and toxiological and clinical aspects. (Eds Larrson A. Orrenius S. Holmgren A. Mannervik B.) Raven press, New York. pg 39-49.

Reed DJ. (1991) Glutathione: toxicoligical implications. Annu. Rev. Pharmacol. Toxicol. **3 0** 603-631.

Reedy CC. Tu CP. Burgess JR. Ho CY. Scholz RW. Massaro EJ. (1981) Evidence for the occurance of selinium-independent glutathione peroxidase activity in rat liver micrisomes. Biochem.Biophys.Res.Comm. **101** 970-978.

Reid GA. Protein targeting in yeast. (1991) J.Gen.Microbiol. **137** 1765-1773.

Reinemer P. Dirr HW. Landenstein R. Schaffer J. Galley O. Huber R. (1991) The three-dimensional structure of class p glutathione S-transferase in complex with glutathione sulfonate at 2.3 Å resolution. EMBO.J. **10** 1997-2005.

Rennenberg H. (1982) Role of glutathione in plant cells. Phytochemistry **21** 2771-2775.

Reyes H. Reisz-Porszasz S. Hankinson O. (1992) Identification of the Ah receptor nuclear translocation protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science **256** 1193-1195.

Rice DA. Aitken LD. Vanderbank GR. Mouv AR. Franklin A. Schimmer BP. and Parker KL. (1989) A cAMP-responsive element regulates expression of the mouse steriod 11β-hydroxylase gene. JBiol.Chem. **264** 14011-14015.

Riezman H. Hay R. Gasser S. Daum G. Schneider G. White C. Schatz G. (1983) The outer membrane of yeast outer mitochondria: isolation of inside-out sealed vesicles. EMBO J. **2** 1105-1111.

Romanos MA. Scorber CA. Clarke JJ. (1992) Foreign gene expression in yeast: a review. Yeast **8** 423-428.

Rosen GM. Rauckman EJ. Ellington SP. Dahlin DC. Christie JL. Nelson SD. (1984) Reduction and glutathione conjugation of N-acetyl-p-benzoquinone imine and two dimethylated analogues. Mol.Pharm.**25** 151-157.

Rushmore TH. and Pickett CB. (1990) Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene: characterisation of a xenobiotic-responsive element controling inducible expresion by phenolic antioxidants. J.Biol.Chem. **265** 14648-14653.

Rushmore TH. King RG. Paulson KE. Pickett CB. (1990) Regulation of glutathione S-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. PNAS. **8 7** 3826-3830.

Rushmore TH. Morton MR. and Pickett CB. (1991) The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J.Biol.Chem. **266** 11632-11639.

Sakai M. Okuda A. and Muramatsu M. (1988) Multiple regulatory elements and phorbol 12-O-tetradecanoate 13-acetate responsiveness of the rat placental glutathione transferase gene. PNAS **85** 9456-9461.

Salo DC Pacifici RE. Lin SW. Giulivi C. Davies KJA. (1990) Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. J.Biol.Chem. **2 6 5** 11919-11927.

Sammuels BL. Murray JL. Cohen MB. Safa AR. Sinha BK. Townsend AJ. Beckett MA. Weichselbaum RR. (1991) Increased glutathione peroxidase activity in a human sarcoma cell line with inherent doxorubicin resistance. Cancer Res. **51** 521-527.

Sanger F. Nicklen S. and Coulson AR. (1977) DNA sequencing with chain terminating inhibitors. PNAS **74** 5463-5467.

Santiago TC. Bettany AJE. Purvis IJ. Brown AJP. (1987) Messenger RNA stability in *Saccharomyces cerevisiae* : the influence of translation and poly (A) tail length. NAR **15** 2417-2429.

Santiago TC. Purvis IJ. Bettany AJE. Brown AJP. (1986) The relationship between mRNA stability and length in *Saccharomyces cerevisiae*. NAR **14** 8347-8360.

Santiago TC. Purvis IJ. Bettany AJE. Brown AJP. (1986) The relationship between RNA stability and length in *Saccharomyces cerevisiae*. NAR **14** 8347-8360.

Satoh K. Kitahara A. Soma Y. Inaba Y. Hamayata I. and Sato K. (1985) Purification, induction and distribution of placental glutathione transferase: A new marker enzyme for preneoplatic cells in the rat chemical hepatocarcinogenesis. PNAS **82** 3964-3967.

Schasteen CS. Krivak BM. Reed DJ. (1983) Similarities in inactivation of glutathione S-transferases by arginine specific chemical modifying agents. Fed.Proc.Am.Soc.Exp.Biol. **42** 2036.

Schauder B. and McCarthy JEG. (1989) The role of bases upstream of the shine-delgrano

region and in the coding rsequence in the control of gene expression in *Escherichia Coli* : translation and stability of mRNA in vivo. Gene **78** 59-72.

Schein CH and Noteborn MHM. (1988) Formation of soluble recombinant proteins in *Escherichia coli* is favoured by lower growth temperature. Biotechnology. **6** 291-295.

Schein CH. (1989) Production of soluble recombinant proteins in bacteria. Biotechnology. **7** 1141-1149.

Schimmer BP. (1980) Cyclic nucleotides in hormonal regulation of adrenocortical function. Adv.Cyclic Nucleotide RES. **13** 118-124.

Schoemaker JM. Brasnett AH. Marston FAO. (1985) Examination of calf prochymosin accumulation in *Escherichia coli* : disulphide linkages are a structural component of prochymosin-containing inclusion bodies. EMBO.J. **4** 775-780.

Schrimer T. and Evans PR. (1990) Structural basis for the allosteric behaviour of phosphofructokinase. Nature **434** 140-145.

Sevanian A. Muskkassah-Kelly SF. Montestruuque S. (1983) The influence of phospholipase A2 and glutahione peroxidase on the elimination of membrane lipid peroxides. Archives Biochem.Biophys. **223** 441-452.

Shah DM. Hironaka CM. Wiegand RC. Harding EI. Krivi GG. Tiemeier DC. (1986) Structural analysis of a maize gene coding for a glutathione S-transferase involved in herbicide detoxification. Plant Mol.Biol. **6** 203-211.

Shen AL. Porter TD. Wilson TE. Kasper CB. (1989) Structural analysis of the FMN domain of NADPH-ctochrome P-450 oxidoreductase by site-directed mntagenesis. J.Biol.Chem. **264** 7584-7589.

Siede W. and Eckardt F. Inducibility of error-prone DNA repair in yeast? (1984) Mut.Res. **129** 3-11.

Siegel FL. Neal TL. Johnson JA. Bertics PJ. Wright LS. (1990) Post-translational modifications of glutathione transferases. In: Glutathione S-transferases and drug resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. pg 47-56.

Sies H. and Moss M. (1978) A role of mitochondrial glutathione peroxidase in modulating mitochondrial oxidations in liver. Eur.J.Biochem. **84** 377-383.

Simula AP. Crichton MB. Black SM. Pemble S. Bligh HFJ. Beggs JD. Wolf CR. (1993) Heterologous expression of drug-metabolising enzymes in cellular and whole animal models. Topics Mol.Pharm. **5** in press.

Sindra BK. Mimnaugh EG. Rajagopalan S. Meyers CE. (1989) Adriamycin activation and free radical formation in human breast tumor cells: protective role of glutathione peroxidase in adriamycin resistance. Cancer Res. **49** 3844-3848.

Sippel HW. (1985) Effect of ethanol on the microsomal glutathione S-transferase activity in glutathione-depleted rat liver. Alcohol **2** 27-30.

Skett P. Chapter 3: (1987) Hormonal regulation and sex differences of xenobiotic metabolism. Progress in drug metabolism **10** Eds Taylor and Francis Ltd, London. pg 85-140.

Smith DB. and Johnson KS. (1988) Single-step purification of polypeptides expressed in *Escherichia Coli* as fusions with glutathione S-transferase. Gene **67** 31-40.

Smith DB. Davern KM. Board PG. Tiu WU. Garcia EG. and Mitchell GF. (1986) M 26 000 antigen of *Schistosoma japonicum* recognised by WEHI 129J mice is a parisite glutathione S-transferase. PNAS **85** 8703-8707.

Soderstrom M. Hammarstrom S. Mannervik B. (1988) Leukotreine C synthase in mouse mastocyma cells: an enzyme distinct from cytosolic and microsomal glutathione transferases. Biochem.J. **250** 713-718.

Song BJ. Gelboin HV. Park SS. Yang CS. and Gonzalez FJ. (1986) Complementary DNA and protein sequences of ethanol inducible rat and human P450s: transcriptional and post-transcriptional regulation of the rat enzyme. J.Biol.Chem. **261** 16689-16697.

Stenberg G. Board PG. Calberg I. Mannervik B. (1991) Effects of site directed mutagenesis on conserved arginine residues in a human class alpha glutathione S-transferase. Biochem.J. **274** 549-555.

Stenberg G. Board PG. Mannervik B. (1991) Mutation of an evolutionary conserved tyrosine residue in the active site of a human class alpha glutathione S-transferase. FEBS **293** 153-155.

Stiles CD. Capone GT. Scher CD. Antoniades HN. Van Wyk. JJ and Pledger WJ. (1979) Dual control of cell growth by somatomedins and platelet derived growth-factor. PNAS **76** 1279-1283.

Studier FW. Rosenberg AH. Dunn JJ. Dubendorff JW. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods in Enzymology **185** Gene expression technology. Article 6 pg 60-89.

Tahir MK. Guthenberg C. and Mannervik B. (1989) Glutathione transferases in rat hepatoma cells. Effects of ascites cells on the isoenzyme pattern in liver and the induction of glutathione transferases in the liver tumour cells. Biochem.J. **257** 215-221.

Tahir MK. Guthenberg C. Mannervik B. (1985) Inhibitors for distinction of three types of human glutathione transferase. FEBS **181** 249-252.

Tamaki H. Kumagai H. Tochikura. (1990) Glutathione S-transferase in yeast: induction of mRNA, cDNA cloning and expression in *Escherichia coli* . Biochem.Biophys.Res.Comm. 172 669-675.

Tamaki H. Kumagai H. Tochikura. (1989) Purification and properties of glutathione transferase from Issatchenkia orientalis. J.Bacteriol. **171** 1173-1177.

Tan KH. Meyer DJ. Belin J. Ketterer B. (1984) Inhibition of microsomal lipid peroxidation by glutathione and glutathione B and AA: role of endogenous phospholipase A2. Biochem.J. **220** 243-252.

Tan KH. Meyer DJ. Coles B. Ketterer B. (1986) Thymine hydroperoxide, a substrate for rat Se-dependent glutathione peroxidase and glutathione transferase isoenzymes. FEBS **207** 231-233.

te Koppele JM. and Mulder GJ. (1991) Steroselective glutahione conjugation by subcellular fractions and purified glutahione S-transferases. Drug Metab.Rev. **23** 331-354.

Telakowski-Hopkins CA. King RG. and Pickett CB. (1988) Glutathione S-transferase Ya subunit gene: identification of regulatory elements required for basal level and inducible expression. PNAS **85** 1000-1004.

Tenant RW. Margolin BH. Shelby MD. Zeiger E. Haseman JK. Spalding J. Caspary W. Resnick M. Stasiewicz S. Anderson B. and Minor R. (1987) Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. Science **236** 933-941.

Tewey KM. Roue TC. Yang L. Halligan BD. and Liu LF. (1984) Adriamycin induced DNA damage mediated by mammalian DNA topoisomerase II. Sceince **226** 466-468.

Thor H. Mirribelli F. Salis A. Cohen GM. Bellomo G. Orrenius S. (1988) Alterations in hepatocyte cytoskeleton caused redox cycling and alkylating quinones. Arch.Biochem.Biophys. **266** 397-407.

Towbin H. Staehelin T and Gordon J. (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. PNAS **76** 4350-4354.

Townsend AJ. Tu CD. Cowan KH. (1991) Expression of human μ or α class glutathione S-transferases in stably transfected human MCF-7 breast cancer cells: effect on cellular sensitivity to cytotoxic agents. Mol.Pharm. **41** 230-236.

Tritton TR. and Lee G. (1982) The anticancer drug adriamycin can be actively cytotoxicity without entering cells. Science **217** 248-250.

Uhlen M. and Monks T. (1990) Gene fusion for the purpose of expression: an introduction. Methods in Enzymology **185** Gene expression technology. Article 12 pg 129-144.

Umeno M. Song BJ. Kozak C. Gelboin HV. and Gonzalez FJ. (1988) The rat P450 2E1 gene: complete intron and exon sequences, chromosome mapping and correlation of development with specific 5'-cytosine demethylation. J.Biol.Chem. **263** 4956-4962.

Ursini F. Valente MM. Ferri L. Gregolin C. (1982) Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degredation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxide BBA **710** 197-211.

Vamvakas S. Dekant W. and Henschler D. (1988). Assessment of unscheduled DNA in a cultured line of renal epithelial cells exposed to cysteine S-conjugates of haloalkenes and haloalkanes. Mut.Res. **222** 329-335.

Vamvakas S. Kordowich FJ. Dekant W. Neudecker and Henschler D. (1988) D.Mutagenicity of hexachloro-1,3-butadiene and its S- conjugate in the Ames test - Role of activation by the mercapturic acid pathway in its nepherotoxicity. Carcinogenesis **9** 907-910.

van Bladderen PJ. Breimer DD. Rotteveel GMT. de Jong RAW. Buijs W. van der Gen A. Mohn GR. (1980) The role of glutathione conjugation in the mutagenicity of 1,2-dibromoethane. Biochem.Pharmcol. **2 9** 2975-2982.

van Bladeren PJ. Breimer DD. van Huijgevoort JATCM. Vermeulen NPE. van der Gen A. (1981) The metabolic formation of N acetyl-S-2-hydroxyethyl-L-cysteine from tetradeutero-1,2-dibromoethane. Relative importance of oxidation and glutathione conjugation in vivo. Biochem.Pharmacol. **30** 2499-2502.

Vercesi AE. Ferraz VL. Macedo DV. Fiskum G. (1988) Ca^{2+} dependent NAD(P)+ - induced alterations of the rat liver and hepatoma mitochondrial membrane permability Biochem.Biophys.Res.Comm. **154** 934-941.

Vernet T. Dignard D. Thomas TY. (1987) A family of yeast expression vectors containing the phage f1 intergenic region. Gene **52** 225-233.

Wallin A. Gerdes RG. Morgenstern R. Jones TW. Ormstad K. (1988) Features of microsomal and cytosolic conjugation of hexachlorobutadiene in rat liver. Chem.Biol.Int. **68** 1-11.

Wallin A. Jones TW. Vercesi AE. Cotgreave I. Ormstad K. Orrenius S. (1987) Toxicity of S-pentachlorobutadienyl-L-cysteine studies with isolated rat renal cortical mitochondria. Arch.Biochem.Biophys. **258** 365-372.

Wallin H and Morgenstern M. (1990) Activation of the microsomal glutathione S-transferase activity by reactive intermediates formed during the metabolism of phenol. Chem.-Biol. Interactions **75** 185-199.

Wang RW. Newton DJ. Pickett CB. Lu AY. (1991) Site-directed mutagenisis of glutathione S-transferase YaYa: non essential role of histidine in catalysis. Archieves of Biochem.Biophys. **286** 574-578.

Wareing CJ. Ph.D. Thesis. University of Edinburgh. (1989).

Watson AJ. and Hankinson O. (1992) Dioxin- and Ah receptor-dependent protein binding to xenobiotic responsive elements and G-rich DNA studied by in vivo footprinting. J.Biol.Chem. **266** 6874-6878.

Watson AJ. and Hankinson O. (1992) Dioxin- and Ah receptor-dependent protein binding to xenobiotic responsive elements and G-rich DNA studied by in vivo footprinting. J.Biol.Chem. **266** 6874-6878.

Wheelan RDH. Wareing CJ. Wolf CR. Hayes JD. Hosking LK. Hill BT. (1992) Over-expression of P-glycoprotein and glutathione S-transferase Pi in MCF-7 cells selected for vincristine resistance in vitro. Int.J.Cancer. **52** 241-246.

Wiekmen A. (1980) Compartmentation and control of amino acid utilization in yeast. In cell compartmentation and metabolic channeling Elsevier/North Holland Biomedical press. Amsterdam. p225.

Wies M. Morgenstern R. Cotagreve IA. Nelson. SD. Moldeus. (1992)N-acetyl-ρ-benzoquinone imine-induced protein thiol modification in isolated rat hepatocytes. Biochem.Pharmacol. **43** 1493-1505.

Wilkinson KD. (1990) Detection and inhibition of ubiquitin-dependent proreolysis. Methods in Enzymology **185** Gene expression technology. Article 32 pg 387-398.

Wolf CR. Berry PN. Nash JA. Green T. Lock EA. (1984) Role of microsomal and cytosolic glutathione S-transferases in the conjugation of hexachloro-1:3-butadiene and its possible relavence to toxicity. J.Pharm.Exp.Ther. **228** 202-208.

Wolf CR. Hayward IP. Lawrie SS. Buckton K. McIntyre MA. Adams DJ. Lewis AD. Scott AR. Smyth JF. (1987) Cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient. Int.J.Cancer **39** 659-702.

Wolf CR. Macpherson JS. and Smyth JF. (1986) Evidence fot the metabolism of mitozantrone by microsomal and cytosolic glutathione S-transferases and 3-Methylcholanthrane-inducible glucuronosyl transferases Biochem.Pharmacol. **35** 1577-1581.

Wolf CR. Seilman S. Oesch F. Mayer RT. and Burke MD. (1986) Multiple forms of cytochrome P450 related to forms induced marginally by phenobarbital: differences in structure and in the metabolism of alkoxyresorufins. Biochem.J. **240** 27-33.

Wolf CR. Wareing CJ. Black SM. and Hayes JD. (1990) Glutahione S-transferases in resistence to chemotherapeutic drugs. In: Glutathione S-transferases and drug resistance (Eds Hayes JD. PickettCB. Mantle TJ.) Taylor and Francis, London. pg 295-308.

Wrange O. Norstedt G. and Gustafsson J-A. (1980) The estrogen receptor in rat liver: quantitative and qualitative analysis by isoelectrical focusing in polyacrylamide gel. Endocrinology **106** 1455-1462.

Xia CL. Cowell IG. Dixon KH. Pemble S. Ketterer B. Taylor JB. (1991) Glutathione transferase π its minimal promoter and downstream cis-acting factor. Biochem.Biophys.Res.Comm. **176** 233-240.

Yamamoto K. and Farber JL. (1992) Metabolism of pyridine nucleotides in cultured rat hepatocytes intoxicated with tert-butyl hydroperoxide. Biochem. Pharmacol. **43** 1119-1126.

Yamazoe Y. Shimada M. Murayama N. Kawano S. and Kato R. (1986) The regulation by growth hormone of microsomal testosterone 6β-hydroxylase in male rat livers. J.Biochem. **100** 1095-1097.

Yamazoe Y. Shimada M. Murayama N. Kawano S. and Kato R. (1987) Suppression of levels of phenobarbital-inducible rat liver cytochrome P450 by pituitary hormone. J.Biol.Chem. **262** 7423-7428.

Yeh GC. Occhipinti SJ. Cowan KH. Chabner BA. Myers CE. (1987) Adriamycin resistance in human tumor cells associated with marked alterations in the regulation of the hexose monophosphate shunt and its response to oxidant stress. Cancer Res. **4 7** 5994-5999.

Yonoha M. and Tampo Y. (1987) Bromosulfophalein abolishes glutathione-dependent protection against lipid peroxidation in rat liver mitochondria. Biochem.Pharmacol. **3 6** 2831-1837.

Yoshida Y and Aoyama Y. (1984) Yeast cytochrome P450 catalysing lanosterol 14-demethlation I: Purification and spectral properties. J.Biol.Chem. **259** 1655-1660.

Yoshimoto T. Soberman RJ. Lewis RA. Austen KF. (1985) Isolation and characterisation of leukotriene C4 synthase of rat basophilic leukemia cells PNAS **82** 8399-8403.

Yoshimoto T. Soberman RJ. Spur B. Austen KF. (1988) Properties of highly purified leukotriene C4 synthase of guinea pig lung. J.Clin.Invest. **81** 866-871.

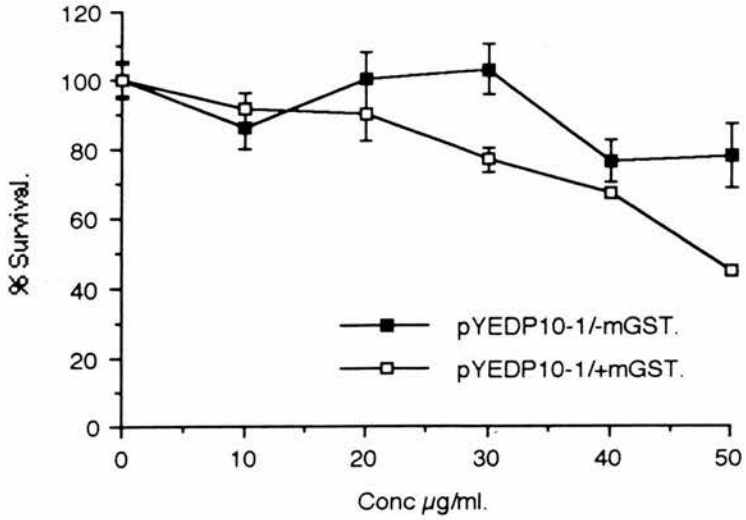
Young RC. Ozols RF. and Meyers CE. (1981) The anthracycline antineoplastic drugs. New England J.Med. **305** 139-153.

Zang P. Graminski GF. Armstrong RN. (1991) Are histidine residues of glutathione S-transferases important in catalysis? J.Biol.Chem. **266** 19475-19479.

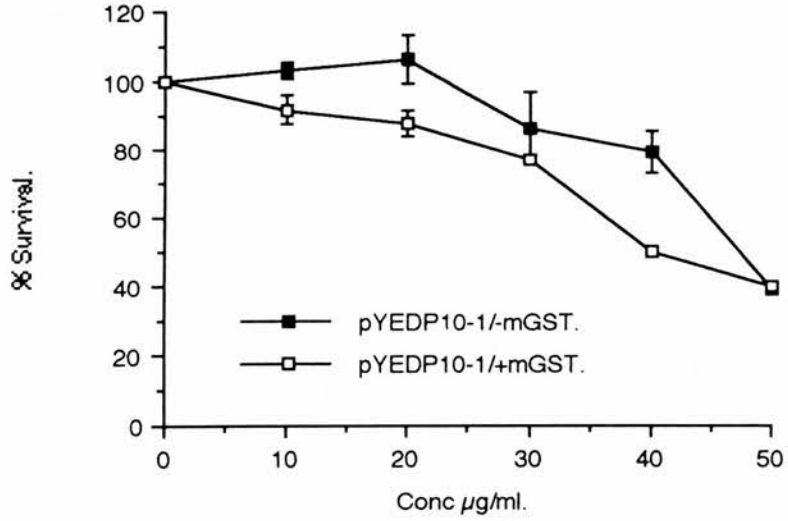
Zaphiropoulos PG. Mode A. Norstedt G. Gustafsson JA. (1989) Regulation of sexual differentiation in drug and steroid metabolism. TIPS **10** 149-153.

Ziegler DM. (1985) Role of reversible oxidation-reduction of enzyme thiol-disulphides in metabolic regulation. Ann.Rev.Biochem. **54** 305-329.

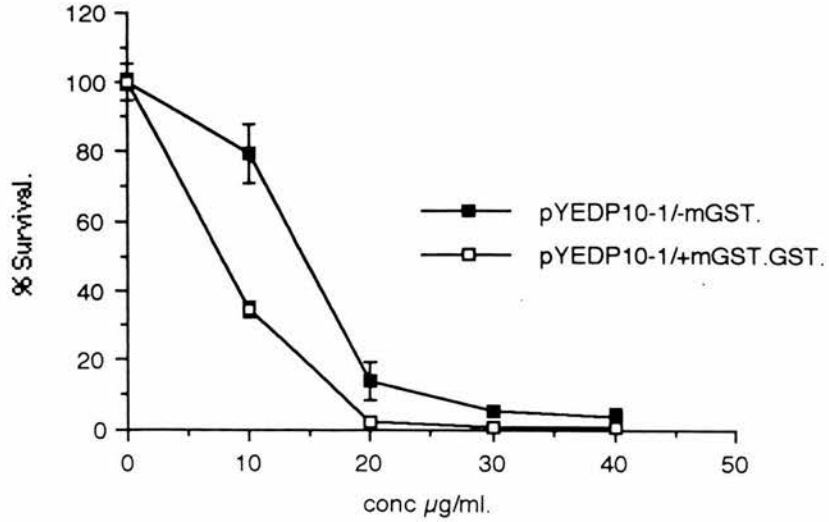
Appendix A: HCBD cytotoxicity experiment conducted at 30oC.



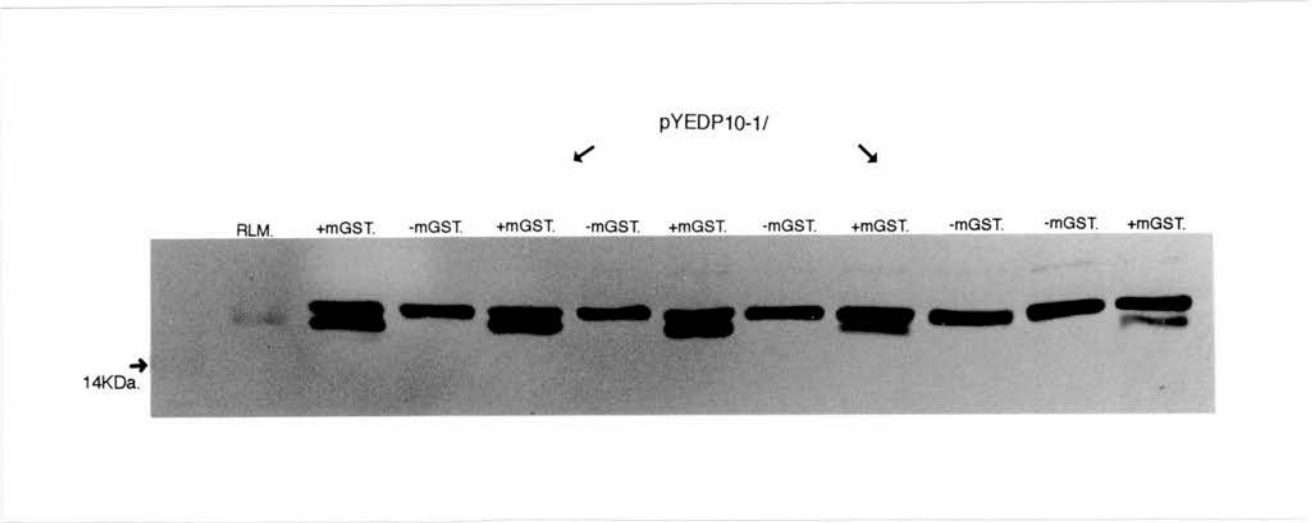
Appendix A: HCBD cytotoxicity experiment conducted at 37oC



Appendix A: HCBD cytotoxicity experiment conducted at 37oC.



Appendix B. Samples of yeast cultures used in cytotoxicity experiments.



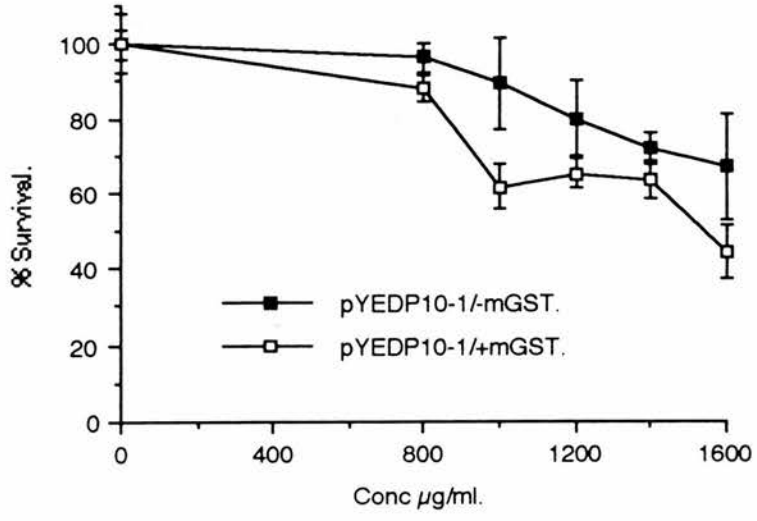
+mGST: rat microsomal GST cDNA in the correct orientation in pYEDP10-1.

-mGST: rat microsomal GST cDNA in the reverse orientation in pYEDP10-1.

50μg of protein from yeast whole cell extracts loaded.

RLM: 5μg of rat liver microsomal protein loaded.

Appendix C: DBE cytotoxicity experiment.



Appendix C: DBE cytotoxicity experiment.